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Title:
Investigating nitrogen transfer between plants in agricultural grassland by using a 15N stable isotope labelling approach

Investigating nitrogen transfer between plants in agricultural grassland by using a ^{15}N stable isotope labelling approach

by

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A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Doctor of Philosophy in the Faculty of Science

School of Chemistry

August 2018

Word count: 77,763

Abstract

The world's population is predicted to reach 9.5 billion by 2050. This will put increasing pressure on already stretched food supplies. Previously, food supply has been increased by the use of synthetic fertilisers, particularly the use of nitrogen (N). However, fertilisers provide an unsustainable source of N, due to high energy demands for production as well as over-application and inadequate matching of fertiliser application to crop demand (synchrony). One solution to this global problem is the use of legumes, such as white clover (*Trifolium repens* L.), which are capable of fixing atmospheric N_2, N can then be supplied to an associated non-legume crop. To date, legume and non-legume cropping systems have seen little application due to a lack of understanding of the unique N-transfer pathway. Three major belowground pathways have been identified: plant exudation, legume decomposition and mycorrhizae associations. A better understanding of the different N-transfer pathways is needed to maximise the benefits of the association and to develop appropriate land-use management strategies, this is addressed by this research.

The research has focused on developing and validating a method for introducing a ^{15}N-label to white clover and following the N-transfer through the plant and soil systems into associated perennial ryegrass (*Lolium perenne* L.). The method developed comprised a split-root labelling technique, enabling $CO(^{15}NH_2)_2$ to be injected into a sand-filled labelling compartment. This allowed substantial ^{15}N enrichment to be achieved, facilitating the investigation of the routing and controls on N-transfer within an agricultural soil. Laboratory experiments revealed that under normal conditions N-transfer from clover to ryegrass, as a proportion of non-legume N derived from the transfer of legume root N ($Ndft_R$), provided on average 2.67% of N. However, similar amounts of N were transferred in the reverse direction (1.98%), showing evidence for bi-directional flow. Incorporation of clover shoots into ryegrass soil, significantly increased $Ndft_R$ (9.34%), whilst, clover exudates are likely to represent about one-third of total N-transfer. Perturbing N-transfer through modifications to the soil biota was shown to increase N-transfer (sterilised soil > weevil addition > fungi addition), although not significantly. Application of compound-specific amino acid (AA) techniques enabled the investigation of whether different N-transfer pathways influenced the distribution of ^{15}N-label within the pool of soil AAs, thereby assessing microbial N assimilation and routing of N. Overall, there was a very low percentage incorporation of the applied ^{15}N-label into individual AAs, although the percentage depended on the individual experiment, with total incorporation into the soil protein pool ranging from 0.1 to 2.4%. The majority of experiments revealed preferential routing into glutamic acid due to its central role within AA biosynthesis, which was seen to be similar to those AAs with the closest biochemical proximity.

A key achievement from this research was the development of a robust repeatable method which allows easy manipulation and the investigation of a range of different treatments on N-transfer from clover-to-ryegrass. New insights into the effect of plant stress through ^{15}N leaf-labelling or clover shoot removal were observed, resulting in significant reductions in the concentrations of soil hydrolysable AAs, questioning the use of the commonly used leaf-labelling technique and the effects of defoliation on N cycling and ecosystem functioning. The results generated from studying different N-transfer pathways revealed the importance of decomposition in N-transfer, revealing the rapid decomposition and N release of clover shoot material. This finding is extremely useful in developing land-use management strategies, where incorporation of clover shoot residues into soil can provide sustainable amounts of N in the short-term, which can improve the synchrony between clover and ryegrass, potentially increasing productivity and sustainability.

Acknowledgements

Firstly, I would like to thank my amazing mother without whom this thesis would not be possible. Not many people would be willing to give up months of their spare time to come and grind up **hundreds** of samples by hand which was just too energy demanding to carry out myself, and who has also read every single last page of this thesis! For this, I promise to look after you in your old age and not shove you into a care home 'too' soon, and if/when I do, I promise to visit at least twice a week! I would also like to thank the rest of my amazing loving family who have supported me through what will always feel like the endless PhD years, especially my husband (Philip) who has stood by me in sickness and in health, through the bad times and the good, to which there have been a lot of the former. Also, to my Dad (aka Mabster) for his loving support and encouragement (I still don't understand why you didn't put me off doing a PhD though!). I would also like to thank Rebecca Veater and my brother (Chris Beach), who have both stood by me for 23+ years and tolerated being dragged into the lab at various weekends to check on the instruments, water or sample the plants, and also to my very dearest Ellie Britton for always being on the end of the phone and drawing me some amazing plants!

I would also like to mention a special dedication to my grandparents (Keith and Pat) whom we have sadly both lost in the last year. I am proud that this thesis contains both of your passions, my Grandad's love for chemistry and my Gran's love of geography and plants. I would like to thank-you so much for all your love and support in everything I have done and inspiring me to travel the world. I miss you both ever so much.

I would also like to thank the large clan of supervisors I have had throughout my PhD for letting me into your labs. Phil Murray for all his expertise on clover, all its pests, and willingly coming to Bristol for meetings. Ian Bull for correcting lots of my funky English, his love for formatting, all the educational meetings and YouTube videos! Thanks are due to the BBSRC for the funding of my research.

I would also like to thank those people without whom the laboratory analysis would not be possible, especially Alison Kuhl for her help, guidance on all things related to amino acids and lots of chats in the lab. I am also very grateful for the liquid nitrogen top ups you did for me and fixing the XP when 'that button' was accidently switched off! And also, to Liz Dixon at North Wyke for running all my bulk analyses. The super human Alice Charteris for some rather in-depth conversations (not about work), answering many weird and wonderful questions about amino acids (about work) even after leaving, and generally being very considerate in helping me out in many other kind ways.

I am also so glad to have met Ili Johari through my PhD, sharing both the lab, office, supervisors, woes and having made such a wonderful friend. I cannot wait to come out and celebrate with you in Malaysia in just a few weeks. I would also like to thank my two Polish friends, Borys Banecki and Hanna Gruszczynska for all our lunch times together and listening to my constant rants!

This would also not be my thesis, without some mention of rabbits whom I have enjoyed many a happy cuddle with, and I hope you enjoyed all the surplus clover and ryegrass! And also, to my cats (Toffee and Liquorice) who can now stop glaring at me as the writing has officially finished and it's time for cuddles!

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Abbreviations

AA	Amino acid
Ala	Alanine
AM	Arbuscular mycorrhizal
Arg	Arginine
Asn	Asparagine
Asp	Aspartate/ aspartic acid
Asx	Deamidated asparagine and aspartate/ aspartic acid
BGN	Below ground nitrogen
BNF	Biological nitrogen fixation
C	Carbon
CEC	Cation-exchange capacity
CMNs	Common mycorrhizal networks
Cys	Cysteine
DCM	Dichloromethane
DDW	Double distilled water
DIN	Dissolved organic nitrogen
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DON	Dissolved organic nitrogen
EA-IRMS	Elemental analyser-isotope ratio mass spectrometer
FID	Flame ionisation detector
GABA	Gamma (γ)-Aminobutyric acid
GDH	Glutamate dehydrogenase
GC	Gas chromatograph(y)
GC-C-IRMS	Gas chromatograph-combustion-isotope ratio mass spectrometry
GC-MS	Gas chromatograph-mass spectrometry
Gln	Glutamine
Glu	Glutamate/ glutamic acid

Glx	Deamidated glutamine and glutamate/ glutamic acid
Gly	Glycine
GS-GOGAT	Glutamine synthetase-glutamine oxoglutarate aminotransferase
	Or glutamine synthetase-glutamate synthase
HFA	Home field advantage
His	Histidine
Hyp	Hydroxyproline
Ile	Isoleucine
IS	Internal standard
LC	Labelling compartment
Leu	Leucine
Lys	Lysine
Met	Methionine
N	Nitrogen
NAIP	*N-acetyl-O*-isopropyl
Ndfr	N derived from rhizodeposition
Ndft	Proportion of N in the non-legume derived from the transfer of legume N
$Ndft_r$	Proportion of N in the non-legume derived from the transfer of legume root N
Nle	Norleucine
NPP	Net primary production
N_r	Reactive nitrogen (all expect N_2)
NUE	Nitrogen use efficiency
NWFP	North Wyke Farm Platform
O	Oxygen
Orn	Ornithine
P	Phosphorus
PAL	Phenylalanine-ammonia lyase
Phe	Phenylalanine

PGPR	Plant-growth promoting rhizobacteria
Pro	Proline
RC	Receiving compartment
RFM	Relative formula mass
RNA	Ribonucleic acid
Ser	Serine
SIP	Stable isotope probing
SMB	Soil microbial biomass
SOM	Soil organic matter
TC	Transfer compartment
Thr	Threonine
Tyr	Tyrosine
Trp	Tryptophan
Val	Valine
WHC	Water holding capacity
%TC	Percentage total carbon
%TN	Percentage total nitrogen

Chapter 1

Introduction

1. Introduction

1.1. Background to food security

The world's population is currently over 7.5 billion people (UN, 2017), of this number, in 2016 there were 815 million chronically undernourished hungry people (FAO et al., 2017). By 2050, it is predicted that the world's population will reach 9.7 billion people (UN, 2017). With this considerably larger population, the number of starving people will increase unless net food production increases by 70-110% to meet rising food demands (Tilman et al., 2011; FAO, 2009a; Ray et al., 2013). Addressing food demand will cause increasing pressure on existing agricultural resources requiring greater crop yields and land. However, increasing food production does not necessarily ensure food security which is of utmost importance. Food security may be defined as "a situation that exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life" (FAO, 2009b). From this definition there are several aspects to food security: food production and availability, access to food (in terms of economic and physical access), food utilisation and stability of supplies over-time (FAO, 2013).

Supply of food to a continually growing world population has previously been addressed through increasing nutrient input to plants. Nutrient management has always been critical to the sustainability of agriculture, but until affordable sources of plant synthetic fertilisers became easily available very few effective options existed (Goulding et al., 2008). Fertiliser application tends to focus on meeting nitrogen (N) demands (Goulding et al., 2008) due to the fact that N is a crucial element in all organisms, required for biochemical processes and the formation of deoxyribonucleic acid (DNA), adenosine triphosphate (ATP), amino acids (AAs), proteins and enzymes (Mattson, 1980). Furthermore, N is the least readily available element to sustain life (Galloway et al., 2003) as the majority of N exists as N_2 gas which is inaccessible to most plants, with N in forms that can be assimilated by plants in short supply. Therefore, N is commonly the limiting nutrient that restricts plant growth in terrestrial ecosystems (Sylvia *et* al., 2005; Gruber and Galloway, 2008; Erisman et al., 2011).

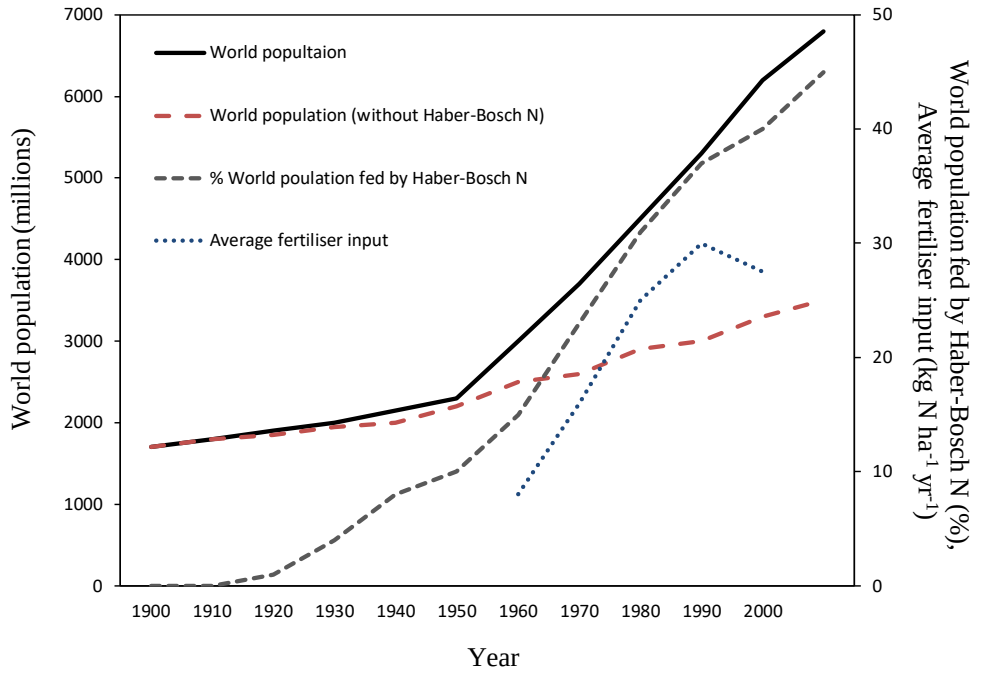


Figure 1.1. World population and the influence of the Haber-Bosch process providing N_r throughout the twentieth century, including estimates for the world population without N_r from the Haber-Bosch process and the percentage of the world population that is supported through the Haber-Bosch process. Increase in average fertiliser use per hectare of agricultural land is also shown. (Adapted from Erisman et al., 2008).

Through the production of synthetic N fertilisers at the beginning of the twentieth century, food production has been able to increase greatly (Gruber and Galloway, 2008), further supporting population growth (Figure 1.1). Food production using synthetic ammonia (NH_3) is the dominant process involving cleavage of the triple bond in molecular N (N_2) *via* the Haber-Bosch process. This uses vast amount of energy (provided by depleting fossil fuel reserves), high temperatures and pressures under a metal catalyst. Essentially the Haber-Bosch process is an artificial method for N_2-fixation, creating reactive nitrogen (N_r) in the form of NH_3 (Galloway et al., 2003; Appl, 2011).

$$N_2 + 3H_2 \rightarrow 2NH_3$$

Equation 1.1

NH_3 from the Haber-Bosch process can be used to produce a variety of other compounds, such as, hydrogen cyanide (HCN), ammonium-sulfate ($(NH_4)_2SO_4$), urea ($CO(NH_2)_2$) and nitric acid (HNO_3) which can be used to produce ammonium nitrate (NH_4NO_3) (Jensen et al., 2011). This can then be used to supply N in a form available to plants (Galloway et al., 2003). According

to Smil (2001) 40% of the people alive today are as a result of the Haber-Bosch process with many more people dependant on this in the future, with recent estimations showing a higher dependence (Figure 1.1). Increases in the number of people supported per hectare of arable land have been made possible by the Haber-Bosch process (1.9 to 4.3 people between 1908 and 2008) (Erisman et al., 2008). Over-application of fertilisers frequently occurs as the economic reward in crop yield offsets the cost of fertiliser, due to restricting the periods when nutrient supply is insufficient to meet crop demands which restricts yields (Crews and Peoples, 2004). However, over-application of fertilisers is unlikely to continue, as in summary, this results in nutrient imbalances, nutrient surpluses, excess of N in some areas, losses of N to the environment; impacting biodiversity, air and water quality and human health. Additionally, N used within fertilisers is produced through industrial N_2-fixation requiring large amounts of energy and high-temperatures to break the triple bond in N_2 and therefore using oil from declining supplies, questioning the sustainability of food production. However, the world's ever-growing population demands more food, meaning it is essential to increase nutrient use efficiency (NUE) and find more sustainable N sources. Furthermore, for sustainable agriculture to be achieved, the correct balance of nutrient management is needed covering all economic, social and environmental aspects (Goulding et al., 2008). Hence, to help address food security, nutrient management of N needs to be improved in order to maintain food production but in a way that reduces the impact on the environment. One way of achieving this is through biological nitrogen fixation (BNF) and synchronising nitrogen release from legumes, such as clover, to meet the demand of associated ryegrass crops in grassland agriculture.

1.2. Terrestrial nitrogen cycling

To help address food supply and a more sustainable supply of N, an understanding of the N cycle is needed. The N cycle involves a sequence of biochemical changes, where a living organism uses N for growth and reproduction, transformation of N then occurs upon death and decomposition and finally it is converted to its original oxidation state through the N cycle (Sylvia et al., 2005). The N cycle is generally mediated by soil microorganisms, but soil macrofauna (nematodes, worms) also play a role in the transformation and recycling of N between its many different forms, as N_r (all forms of N except N_2) exists as inorganic N (NH_4^+, NO_3^-, NO_2^-) and organic N ($CO(^{15}NH_2)_2$, AAs) (Sylvia et al., 2005); fundamentally the N cycle maintains a pool of biologically available N (Nelson and Cox, 2013). There are five key

processes which take place in soils involving the transformation of N: N_2-fixation, mineralisation (conversion of organic N to inorganic N), nitrification (conversion of NH_4^+ to NO_2^- then NO_3^-), denitrification (conversion of NO_3^- to N_2O then N_2) and immobilisation or assimilation (uptake of inorganic N by microbes and other soil heterotrophs) (Figure 1.2) (Sylvia et al., 2005; Paul., 2007; van Elsas et al., 2007).

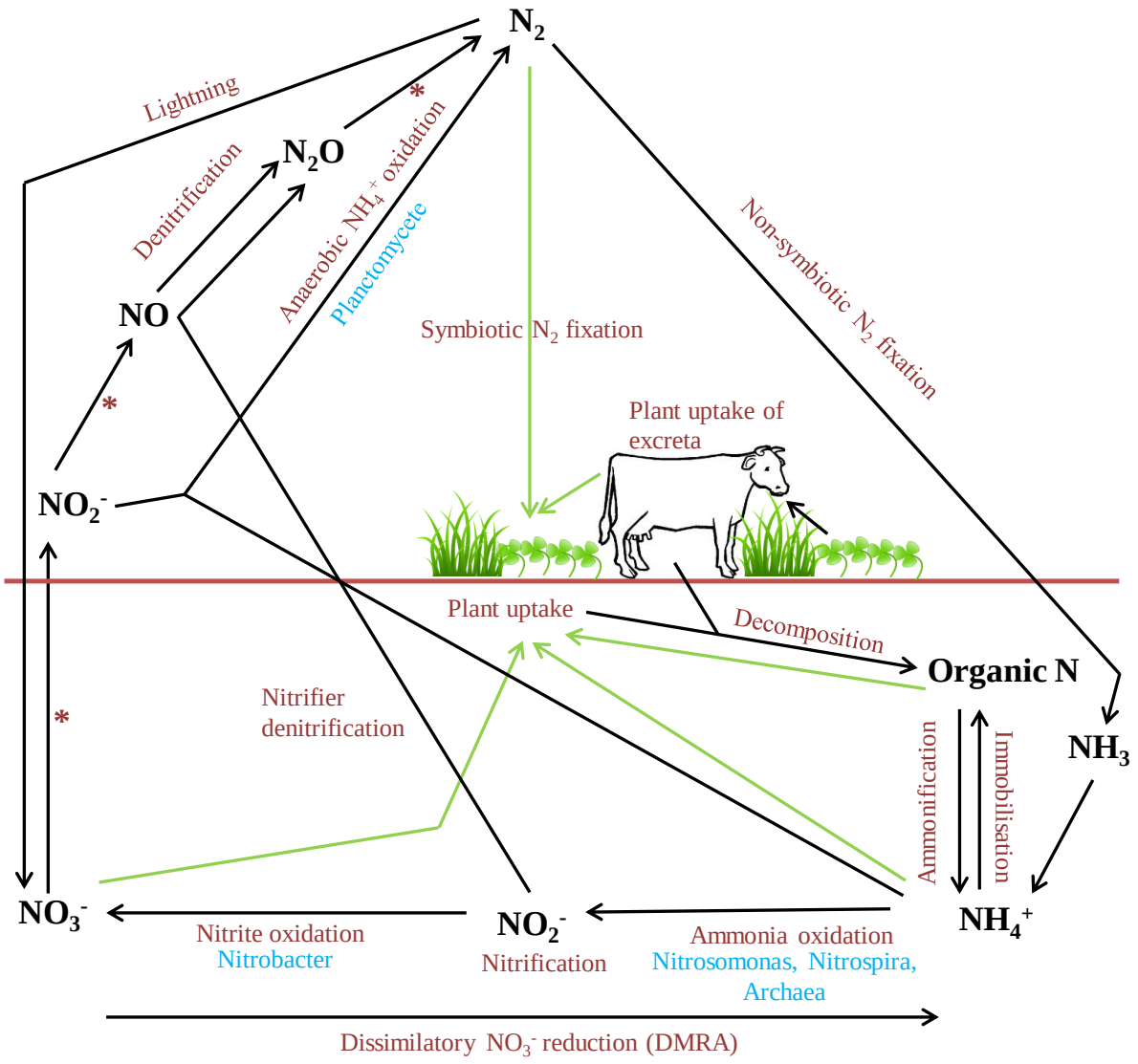


Figure 1.2. The nitrogen cycle, showing the conventional view alongside recent developments in new processes and players which are involved (Leininger et al., 2006; Shaw et al., 2006; Humbert et al., 2009). Red text shows the processes involved, blue text shows the main organisms involved in the process and green arrows illustrate uptake pathways by plants. * denotes the denitrification pathway from NO_3^- to N_2 *via* intermediate steps. (Adapted from Sylvia et al., 2005)

The N cycle is unique compared to other biogeochemical cycles since 99.96% of all N is atmospheric N_2, there are only a few natural processes in the terrestrial N cycle that can convert this vast pool of N, either through BNF of free-living diazotrophic bacteria (non-symbiotic) or through rhizobial association with legume plants (symbiotic), or lightning conversion to NO_3^-. It is estimated that inputs into the biosphere each year from BNF are 90 Tg N (with a further 33 Tg N added through BNF associated with agriculture), lightning contributes 5 Tg N and human controlled inputs through the Haber-Bosch process are 85 Tg N (Erisman et al., 2005). The transformations between inorganic forms of N have been widely studied, however, much less is known about the transformations and importance of organic N, with organic N being the major fraction of N in soils, far outweighing that of inorganic N in most soils (Mengel, 1996; Schulten and Schnitzer, 1998; Friedel and Scheller, 2002; Jones and Kielland, 2012). Furthermore, approximately only half of the compounds present in organic N have been identified, this includes: proteins, AAs, microbial cell-wall polymers, amino sugars and nucleic acids (Sylvia et al., 2005), however, AAs and sugars make up the clear majority of organic N, which contribute around 30-40% of total soil N (Smith et al., 1993). The past decade has seen a dramatic expansion in our understanding of N-cycle processes (Francis et al., 2007), however, there are still many shortfalls in our knowledge of quantifying and characterising N processes and the factors which regulate N-cycling, partly due to the control that biotic interactions have on a range of processes (van Groenigen et al., 2015). Understanding how to meet the shortfalls in N demand in a sustainable way is crucial for agriculture and food security due to N's fundamental role (Vitousek et al., 1997).

1.3. Plant uptake of N

The N cycle is fundamental to making N available to plants, as plants can only take up N in certain forms as well as recycling anthropogenic inputs of fertilisers and manures (Sylvia et al., 2005). However, plants can also shape the N-cycle through exudates, which mediate energy and nutrients to microbes, and by altering conditions which regulate microbe activity, resulting in community modifications and influencing N transformations (van Groenigen et al., 2015; Coskun et al., 1017). Approximately 2% of all terrestrial N is within plants (Sylvia et al., 2005) with sufficient N availability determining the production of high-quality protein-rich food with all human nutritional N requirements being obtained directly or indirectly from plants. Plants can acquire the N they need for growth and development through several different sources:

(i) application of $NH_4^+$and/or NO_3^- or $CO(NH_2)_2$ fertiliser,

(ii) release of N compounds through organic matter decomposition and mineralisation of N in soil organic matter (SOM),

(iii) atmospheric deposition and conversion of N through natural processes, i.e. lightning,

(iv) N release from atmospheric N_2-fixation by bacteria in symbiosis within a plant (BNF) or free- living diazotrophic symbiosis BNF (Vance, 2001; Chapman et al., 2006).

1.3.1. Plant acquisition of inorganic N

As already discussed, the importance of soil inorganic N within ecosystems has been well characterised compared to that of soil organic N, as compounds can readily be separated and measured. Inorganic N plays an important role serving as: substrates, metabolic intermediates and alternative electron acceptors as well as being the product of many biological N transformations (Sylvia et al., 2005). Furthermore, approximately 99% of all organic N on the Earth is derived from NO_3^- assimilation (Heldt, 2005). It is well acknowledged that plant roots take up low molecular mass N compounds in the form of NH_4^+ and NO_3^- (Paungfoo-Lonhienne et al., 2008) with net mineralisation being a key process in soil N cycling providing N to plants (Schimel and Bennett, 2004). Both NH_4^+ and NO_3^- have advantages and disadvantages in their use as an N source to plants (Lewis, 1986) with plants themselves having a preference towards NH_4^+ or NO_3^- uptake (Hageman, 1984; Forde and Clarkson, 1999; Boudsocq et al., 2012).

In well-aerated non-acidic soils, NO_3^- is the main source of N to plants through the activity of nitrifying bacteria (Lewis, 1986). NO_3^- may be assimilated in the roots or shoots, however, commonly in the early stages of growth NO_3^- is assimilated in the roots, and later when plants are fully grown assimilation primarily occurs in the leaves for herbaceous plants. However, legumes tend to mostly assimilate NO_3^- in the roots (Heldt, 2005). Two NO_3^- transport systems are shown to co-exist, varying from low affinity to high affinity (Heldt, 2005; Tsay et al., 2007). NO_3^- absorption is influenced by several factors, such as: energy-rich compound availability, soil temperature, pH and presence of NH_4^+ ions in the root medium (Lewis, 1986). Once absorbed by plants NO_3^- can be temporarily stored in the vacuole (Heldt, 2005) or is reduced *via* assimilatory NO_3^- reduction. Through this procedure NO_3^- is firstly reduced to NO_2^- by nitrate reductase in the cytosol, and then to NH_4^+ in the chloroplasts by nitrite reductase (Figure

1.3, Equation 1.2-1.3). Plant uptake of NO_3^- has the advantage of increasing the absorption of K^+, Ca^{2+} and other cations, which can lead to an overall more even charge balance in plants. However, there are disadvantages of using NO_3^- as it must be first converted to NH_4^+ to be used by the plant which is an energy demanding process, as well as the fact that absorption is energy dependent requiring ATP. Furthermore, the greatest disadvantage of NO_3^- is that it is mobile, meaning it is easily leached from soils (due to its negative charge resulting in non-adsorption onto soil particles) therefore it is less available to plants (Lewis, 1986; Brady and Weil, 2008; Boudsocq et al., 2012). However, it can also be seen that this high mobility allows rapid root uptake (Boudsocq et al., 2012).

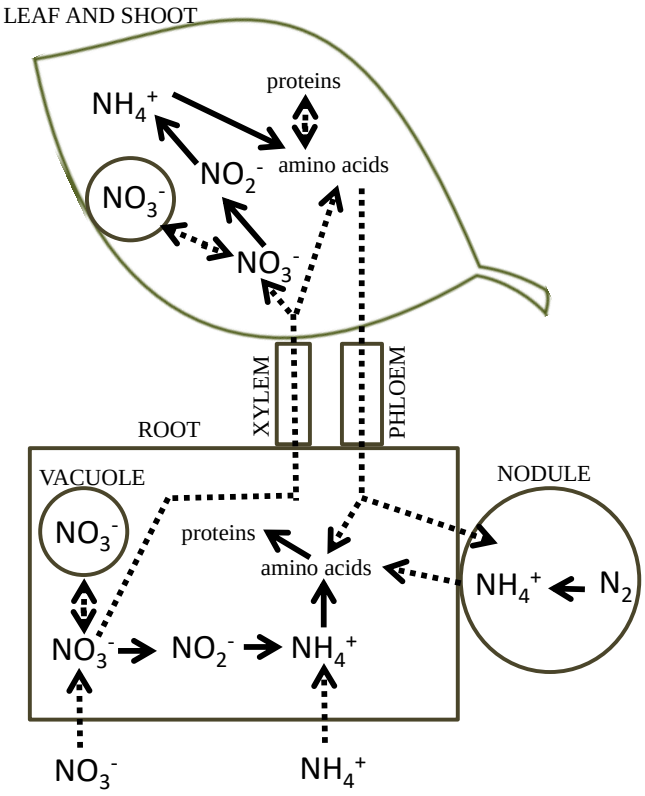


Figure 1.3. Simplified diagrams of N flow within plants (adapted from Lea and Leegood, 1999), in legumes glutamine, asparagine and ureides are the primary nitrogenous compounds transported through the plant (Temple et al., 1998).

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$$

Equation 1.2

$$NO_2^- + 6H^+ + 6e^- \rightarrow NH_4^+ + 2OH^-$$

Equation 1.3

In plants as well as bacteria, if NH_4^+ (produced by reduction of NO_3^- or absorbed from soil) has a plentiful supply it is typically incorporated or assimilated into AAs, mainly glutamine (Gln) and asparagine (Asn) (collectively called amides), through a reaction with 2-oxoglutaric acid (an intermediate from C metabolism, otherwise known as α-ketoglutanic acid) through a reversible reaction involving the enzyme glutamate dehydrogenase (GDH) to form glutamate (Glu). However, the GDH pathway is not only reversible but it also has a low affinity for NH_4^+, leaving organisms to frequently exist in low NH_4^+ conditions (Postgate, 1998) meaning this process is inefficient (Figure 1.4). Furthermore, GDH exists in all organisms (e.g. bacteria and eukaryotes), therefore this reaction is common to all organisms, although some bacteria lack the required enzyme (Santero et al., 2012).

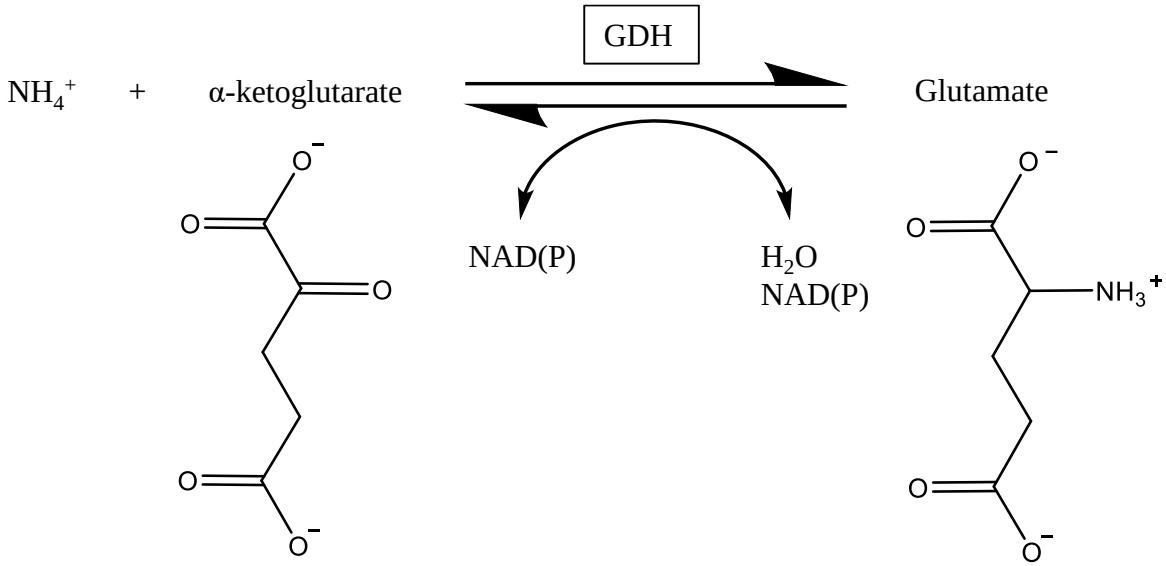


Figure 1.4. Ammonium assimilation *via* the GDH pathway in all organisms, enzymes are shown in boxes.

In conditions of low NH_4^+ availability, NH_4^+ is assimilated into organic compounds *via* the glutamine synthetase-glutamine oxoglutarate aminotransferase (GS-GOGAT) pathway (Figure 1.5), this is thought to be the major pathway (Temple et al., 1998). NH_4^+ is directly incorporated into Gln, using glutamate (Glu) as a substrate, catalysed by the enzyme glutamine synthetase (GS), which is efficient at picking up NH_4^+ (Postgate, 1998). The amide-N of Gln can further be transferred to a 2-oxoglutaric acid by glutamate synthase (GOGAT) to Glu (Lewis, 1986; Forde and Woodall, 1995). Through this reaction pathway organisms can convert one Glu molecule into two, allowing NH_4^+ to be assimilated efficiently at the expense of ATP (Postgate, 1998). This pathway is estimated to be 18% higher in energy requirement than the GDH

pathways (Helling, 1994). The primary source of all of the other AAs found in plants as well as bacteria originates from Gln through transamination reactions between Glu and corresponding α-keto acids (Lewis, 1986; Forde and Woodall, 1995; Forde and Lea; 2007), hence the amide-N in Gln is the primary source of N for all other AAs. Transamination (or aminotransfer) reactions is where the α-amino group of an AA replaces the α-keto (2-oxo) group of an α-keto organic acid, the reaction is catalysed by transaminases (or amino-transferases, TA). The rate of NH_4^+ uptake is dependent on a good carbohydrate supply much more than NO_3^- uptake, due to the fact that NH_4^+ must be immediately incorporated into an organic amino molecule as NH_4^+ build-up is toxic; this occurs exclusively in the roots (Lewis, 1986; Temple et al., 1998). Although NH_4^+ uptake overall is seen to be a lower energy demanding process than NO_3^- uptake and assimilation. Two biosynthetic routes appear to exist for NH_4^+ production: energy dependent and independent (Lewis, 1986; Boudsocq et al., 2012). Both GDH and GS-GOGAT pathways are the only means of incorporating inorganic N into C backbones, representing fundamental steps within all cell metabolisms (Santero et al., 2012).

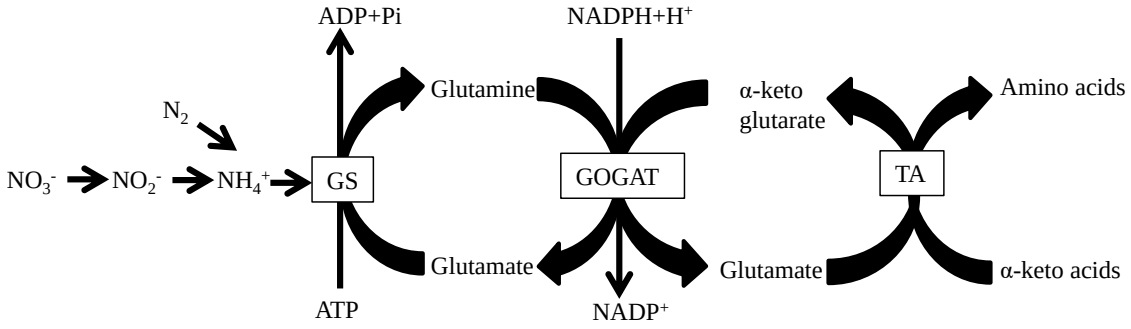


Figure 1.5. Ammonium assimilation *via* GS-GOGAT pathway in all organisms. Enzymes are shown in boxes: GS glutamine synthetase, GOGAT glutamate synthase, TA transaminases. Adapted from Lewis (1986), Dixon and Wheeler (1986), Lea (1997) and Lea and Leegood (1999).

Synthesis of AAs mostly takes places in plant chloroplasts, the final product of NO_3^- assimilation can be regarded as the sum total of the AAs produced. However, plant species and metabolic conditions determine the pattern of AAs synthesised, commonly Gln and Glu represent the majority of synthesised AAs (Heldt, 2005). Importantly, the C skeletons from which all AAs are synthesised comes from CO_2 assimilation, whilst the different C backbones (intermediates) originate from either glycolysis, pentose phosphate pathway or *via* the citric acid cycle (Berg et al., 2015). However, the most important precursor for AA synthesis is 3-

phosphoglycerate, which leads to the production of 13 AAs (Figure 1.6) (Heldt, 2005). N metabolism slightly varies from that of C, where Glu and Gln are the N donors for a wide range of molecules, where this is regulated through the enzyme glutamine synthetase (Nelson and Cox, 2013).

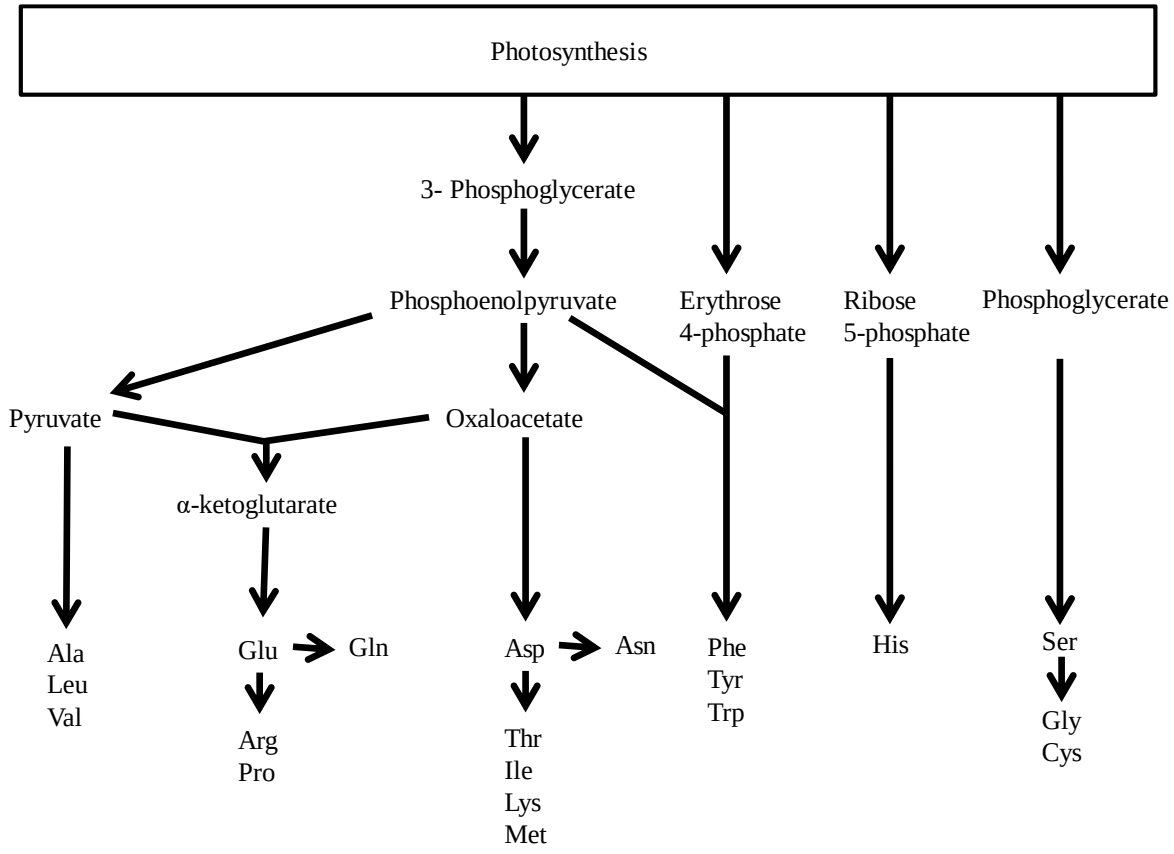


Figure 1.6. Origin of C skeletons and main precursors for the synthesis of AAs. (Adapted from Heldt, 2005)

1.3.2. Plant acquisition of organic N

Until relatively recently it was assumed that plants only use low molecular mass inorganic N and generally compete poorly for N against microbes (Schimel and Bennett, 2004; Weigelt et al., 2005). However, it has now been shown in a number of studies that plants are able to assimilate AAs, peptides, proteins, and other low molecular weight organic N compounds, in competition with the soil microbial community (Chapin et al., 1993; Nasholm et al., 1998, 2000; Henry and Jefferies, 2002; Weigelt et al., 2005; Paungfoo-Lonhienne et al., 2008; Hill et al., 2011; Jones and Kielland, 2012) (Figure 1.7).

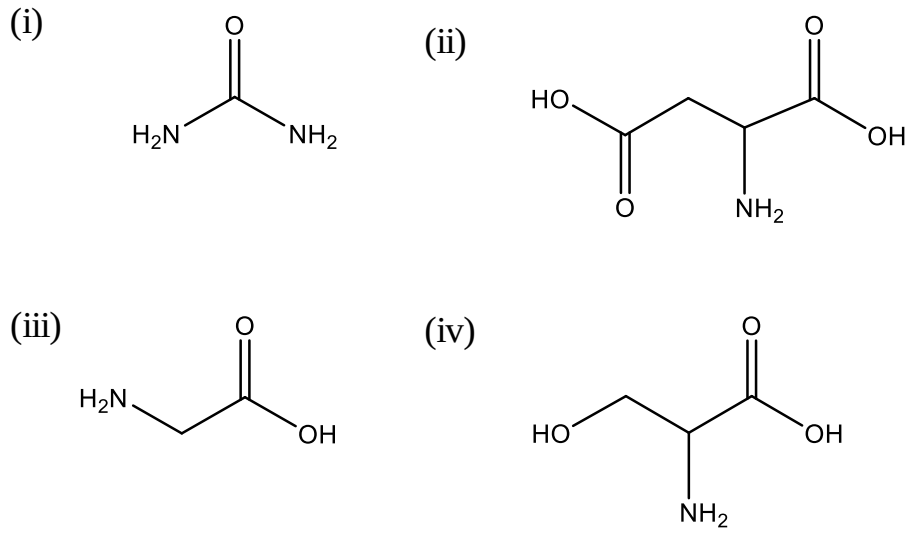


Figure 1.7. Some examples of organic N taken up by plants, (i) urea, (ii) aspartic acid, (iii) glycine, and (iv) serine.

Demonstrating the significance of organic N to plants, globally, urea ($CO(NH_2)_2$) is the most commonly used fertiliser, with usage doubling in the past 10 years alone (Glibert et al., 2006); commercial production through the Haber-Bosch process began in the 1920s (Smil, 2001). However, following the application of $CO(NH_2)_2$ to soils, it is rapidly hydrolysed by the enzyme urease which can be produced by most microorganisms and plants, to NH_3 and carbamate (NH_2CO_2H). The latter can then be decomposed to another molecule of NH_3 and carbonic acid (H_2CO_3) (Sumner et al., 1931; Watson et al., 1994; Mobley et al., 1995; Gill et al., 1999; Equation 1.4-1.5) and then potentially to NO_3^-.

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{Urease}} NH_3 + NH_2CO_2H$$

Equation 1.4

$$NH_2CO_2H + H_2O \rightarrow NH_3 + H_2CO_3$$

Equation 1.5

Therefore, application of $CO(NH_2)_2$ to the rhizosphere can result in $CO(NH_2)_2$, NH_4^+ and NO_3^- co-existing in soils (Mérigout et al., 2008). Urease inhibitors are frequently applied to soils to slow the rate of $CO(NH_2)_2$ hydrolysis, reducing problems which can be associated with application (i.e. NO_3^- leaching). Furthermore, this can enable plants to uptake $CO(NH_2)_2$, which they are capable of doing, either through their roots or shoots (Hine and Spent, 1988; Krogmeier et al., 1989; Gerendás et al.,1998; Gill et al., 1999; Mérigout et al., 2008; Trépanier

et al., 2009; Trenkel, 2010); several mechanisms for its direct acquisition have been described (Pinton et al., 2016).

It has been demonstrated that plants have mechanisms which can transport AAs into their roots from the soil, in addition, studies have identified a variety of AA transporters (Jones et al., 2005; Rentsch et al., 2007). Jones et al. (2005) proposed that the main function of root positioned AA transporters is to recapture AAs which are lost through exudation and their minor role is to transport AAs that are released from the SOM. Conversely to this opinion, Chapin et al. (1993) showed that some plants have a preferential uptake of AAs compared to inorganic N; for example, this was shown for the non-mycorrhizal artic sedge (*Eriophorum vaginatum*). A similar finding has also been found within grassland species, where a grass species gradient was studied (fertilised, productive pastures to extensive, low productivity pastures) establishing marked differences in the uptake preference of different chemical forms of N of varying complexity between inter-species of grass (Weigelt et al., 2005). Further to this, plants have additional mechanisms which they depend on to help facilitate AA and protein uptake, for example, forming mutualistic symbioses with mycorrhizal fungi (Paungfoo-Lonhienne et al., 2008). When ryegrass (*Lolium perenne* L.) is colonized by arbuscular mycorrhizal (AM) fungus, uptake of the AAs aspartic acid (Asp) and serine (Ser) is increased compared to control plants. However, when the biomass of these plants raised on different nutrient solutions was compared, there was no difference between the NO_3^- and Ser feed plants but the biomass of the Asp fed plants was significantly lower (Cliquet et al., 1997). Paungfoo-Lonhienne et al. (2008) showed that plants do not need mycorrhizal fungi for protein uptake, furthermore, identifying possible mechanisms which could enable plants to do this. It was suggested that proteolytic enzymes are exuded from the roots which allow the digestion of proteins at the surface of the root, but maybe as well at the root cortex apoplast and/or root cells. Intact proteins are then possibly taken up by endocytosis following which proteins are catabolized. However, it was found that if N was solely provided through protein then plant growth was not as well supported as through inorganic N. Additionally, when the two sources are used in conjunction with one another, as in natural systems, protein is seen to supplement plant N demand.

Nonetheless, the role of organic N in the agricultural environment is still very much debated. Studies have shown that in grasslands, glycine (Gly) has been shown to be taken up directly (Nasholm et al., 2000; Thornton, 2001; Bardgett at el., 2003). Other studies have suggested

that the main route for plant N uptake in agricultural systems is through mineral N following microbial mineralisation due to fast microbial turnover (Owen and Jones, 2001; Bardgett et al., 2003). Although, it has been shown that more soil organic N is captured by grassland plant roots at unimproved sites where AAs are the dominant soluble N form (Bardgett et al., 2003). Owen and Jones (2001) studied the competition for AAs between microorganisms and wheat roots (*Triticum aestivum* cv. Altas) finding that the wheat roots could only capture 6% of the AAs with the remainder captured by the soil microbial community. Several reasons have been suggested for this finding within the agricultural environment: higher NO_3^- concentrations in agricultural soils, slow movement through soil of AAs compared to NO_3^-, rapid turnover of AAs by microbes alongside the low competitive ability of plant roots to capture AAs. Furthermore, as concluded by Owen and Jones (2001), "…if amino acids contribute little to the overall N budget of a plant, why do roots have and operate amino acid transporters?" Furthermore, organic N uptake could be seen to have further advantages to the plant over that of inorganic N, due to satisfying both C and N requirements (Geisseler et al., 2010; Franklin et al., 2017), as well as having an advantage within environments where soil organic N is high (Moe, 2013).

Nevertheless, studies are changing our view on the number of different N sources that can be exploited by plants and the reliance of plants on microbes and soil fauna for organic matter breakdown (Paungfoo-Lonhienne et al., 2008). While it has been shown that plants do take-up AAs, the evidence demonstrating the overall contribution of AAs to the N budget of a plant and of this as a major pathway for N acquisition as well as its ecological significance is still lacking (Owen and Jones, 2001; Jones et al., 2005). However, this is likely to depend on many combined factors within a soil's ecosystem, as such: the relative contribution of a range of N solutes in the soil solution, available inorganic N soil reserves, organic N mineralisation rates and competition from soil microorganisms (Owen and Jones, 2001) as well as the growing amount of evidence for species-specific preferential uptake of different chemical forms of N (Bardgett et al., 2003). Furthermore, the cycling of soil organic N remains poorly understood, especially its active cycling through the soil system and transfer between inorganic and organic N pools, due to its complexity and heterogeneity (Charteris et al., 2016); AAs are also recognised as a key intermediary in the soil N-cycle (Moe, 2013). Therefore, greater understanding at the molecular level is needed to elucidate the nature and rates at which organic N compounds are made available for plant uptake.

1.3.3. Plant acquisition of atmospheric N_2

Only certain bacteria and archaea (diazotrophs) are able to carry out BNF, these can form an interaction with plant roots on a number of levels. For example: non-symbiotic associations between free-living diazotrophs (such as *Azotobacter* spp), associative symbioses forming partnerships between *Frankia* spp. or cyanobacteria in trees and woody scrubs to the more complex symbiotic associations between *Rhizobium* spp. and legumes (Peoples and Craswell, 1992; Bowsher et al., 2008; Nelson and Cox, 2013). The more complex interaction enables host plants and the bacterium to communicate on a molecular level and share physiological functions (Sylvia et al., 2005).

BNF is a crucial process within agriculture due to its capability of fixing substantially greater amounts of N than synthetic processes (Burris and Roberts, 1993: Sylvia et al., 2005). However, both of these processes are energy demanding due to the two atoms of nitrogen being joined together by a very stable triple bond (N≡N). To break the bond 945 kJ or 226 kcal per mole is required with further energy being required to obtain the hydrogen necessary to reduce N_2 to two ammonia molecules. In BNF, the reaction must occur at biological temperatures and pressures, therefore, the high activation barrier is overcome by binding and hydrolysis of ATP:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

Equation 1.6

To further overcome the kinetic challenges seen in BNF, the reaction requires an enzyme with multiple redox centres to be produced by microbes, the nitrogenase complex. The nitrogenase complex may be set apart from other complexes as it is formed of 2 proteins: a dinitrogenase reductase (or Fe protein) providing the electrons with high reducing power, and dinitrogenase (or MoFe protein) which uses the electrons to reduce N_2 to NH_3 coupled with the hydrolysis of ATP (Figure 1.8). In most organisms which can carry out BNF the electrons are provided from ferredoxin (Fd) which is generated by oxidative processes or alternatively flavodoxin may be used (Berg et al., 2015). This complex may also be set apart as: it is destroyed by O_2, activation requires Mg^{2+}, when functioning it converts ATP to ADP, however, ADP inhibits its functioning. Furthermore, the nitrogenase complex may reduce other molecules containing triple bonds other than N_2 (Postgate, 1978; 1998; Smith, 1982; Sylvia et al., 2005).

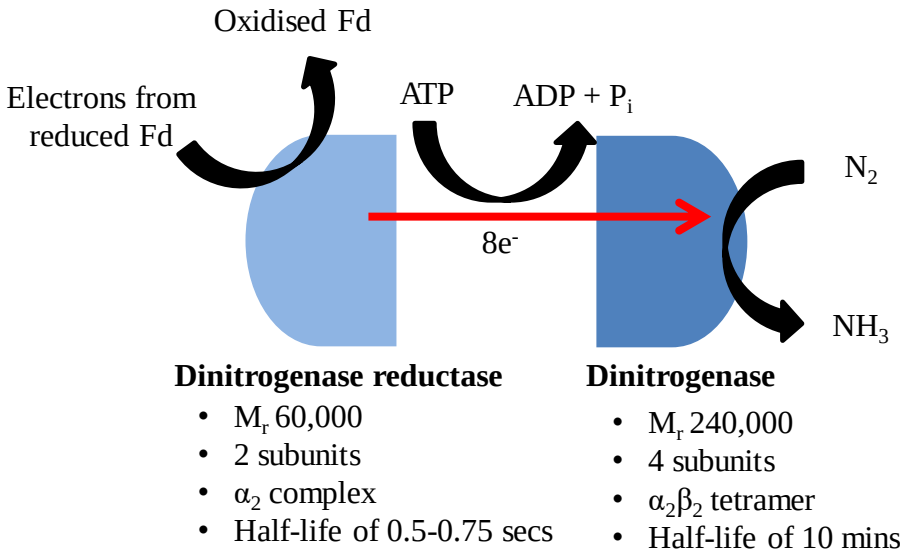


Figure 1.8. Nitrogenase complex involved in BNF (Adapted from Sylvia et al., 2005; Berg et al., 2015)

1.3.3.1. Symbiotic Biological Nitrogen Fixation (BNF)

A mutualistic symbiosis may exist between prokaryotic organisms capable of N_2-fixation and a eukaryotic photosynthetic host where both organisms may derive a benefit. The prokaryotic organism is capable of converting N into a form that the host can utilise, thus allowing it to grow in environments where soil N availability is low. The host provides the micro-symbiont with a protected environment as well as energy, phosphate and other nutrients to enable the prokaryotic to fix N_2, which they cannot do independently (FAO, 1984), typically this association is found within legumes and *Rhizobium* spp. Legumes were recognised to have an importance in agriculture in early history and were used in crop rotations during the Roman times, however, it was not until the 1800s that the presence of nodules on legumes were identified to carry out N_2-fixation with their associated rhizobial bacteria and that the *Rhizobium* bacteria was isolated and described (Postage, 1971; Lewis, 1986; Sylvia et al., 2005). The Leguminosae family comprises of approximately 20,000 plant species in about 650 genera, which can further be divided into 3 subfamilies (Papilionoidae, Mimosoidae, Caesalpinoidae), not all species form associations with *Rhizobium* and only 15% of the species have been studied (FAO, 1984; Dixon and Wheeler, 1986; Postage, 1998). Legumes (family *Fabaceae*) are the most commonly recognised N_2-fixing symbioses due to their importance as a food source, many of our important food crops belong to this family and contain high levels of protein, they include: alfalfa (*Medicago sativa*), clover (*Trifolium spp)*, peas (*Pisum spp*),

beans (*Phaseolus spp*), lentils (*Lens spp*) and lupins (*Lupinus spp*) (Paul, 2007). Legumes are capable of fixing a considerable amount of N, for example: Alfalfa (*Medicago sativa*) 150-250 kg N $ha^{-1}yr^{-1}$, Clover (*Trifolium pratense* L.) 100-150 kg N $ha^{-1}yr^{-1}$ and locust tree (*Robina* spp.) 75-200 kg N $ha^{-1}yr^{-1}$ (Brady and Weil, 2008), therefore, showing their potential as an alternative to industrially produced N fertilisers (Jensen 1994a; Nicolardot et al., 1995). Furthermore, the use of legumes has the capacity to restore disturbed or impoverished soils, reduce the contamination of NO_3^- in groundwater, provide excellent cover crops, green manures and can be used by livestock as forage crops (Sylvia et al., 2005). It is assumed within agriculture that the N_2-fixing plant will meet all or part of its N requirements from BNF, with any surplus being transferred to the soil to later benefit other plants (FAO, 1984; Peoples and Craswell, 1992). However, this may not be the case as N_2-fixation is dependent on many factors, covering: physical, environmental, nutritional and biological influences (Gibson et al., 1982; Chalk, 1991) therefore, careful consideration needs to be made if legumes are to be used as the main input of N in agriculture.

For symbiotic BNF to take place, rhizobial bacteria need to firstly infect their host, which results in the formation of root or stem nodules (Figure 1.9). Rhizobia occur in most soils but effective nodulation production does not always occur due to there either being too few rhizobia present or the rhizobia being unable to work effectively with the legume as a degree of specificity exists (FAO, 1984; Lewis, 1986). There are several different mechanisms for infection: (i) root hair penetration and infection thread formation, this tends to occur in clover and beans, (ii) entry through wounds or lateral root emergence sites, this tends to be found in peanuts (*Arachis hypogaea*) and the pasture legume *Stylosanthes*, (iii) root primordia penetration which can be found on the stems of plants such as *Sesbania* (Sylvia et al., 2005).

Figure 1.9. Root nodules on white clover (*Trifolium repens*) studied in this project.

In brief, once rhizobia bacteria identify the "correct" host through the production of lectin proteins which bind to certain sugar residues on the root hair wall, rhizobia can then synthesise compounds causing the root hair to deform and curl. Following infection, rhizobia are then able to enter and the root hair wall folds back on itself allowing the infection thread to enter the inner cortex of the root. Bacteria are able to enter the inner cortex and are released into the cytoplasm where they multiply. This further results in cell division in the host's root cells and enlargement, resulting in visible changes in the host between 5 to 12 days, where the host cells become proliferated and nodules form (FAO, 1984; Lewis, 1986; Sylvia et al., 2005; Ledgard and Steele, 1992). Two different types of nodule exist: determinate and indeterminate. Determinate nodules are rounded and do not have a noticeable meristematic region, such as in soybean and *Phaseolus*. Determinate nodules produce Glu and Asp which are then used to produce purines, for example xanthine, which is then later converted into ureides, allantoin and allantoic acid. Legumes transport N from the root nodules in either one of these forms, as not all legumes can produce ureides, this pathway is mainly restricted to *Phaseoleae* and *Desmodieae* within the *Papilionoideae* subfamily (Unkovich et al., 2008). By contrast, indeterminate nodules have a pronounced meristematic region and are elongated in shape, such as in peas, clover and medics (*Medicago spp*). Indeterminate nodules produce NH_3, which is then transported to the host cell and is converted to Gln. Gln is then converted following the AA biosynthesis pathway to Asn (Figure 1.5-1.6); Asn is then exported to the shoot (Sylvia et al., 2005). Furthermore, the colour of the nodules is a determinant factor as to whether the nodules are active (effective) or not active (ineffective). Ineffective nodules being white or greenish brown as a result of either the symbiosis being ineffective or the nodule senescing; however, there is some variation in colour. Effective nodules which are active in N_2-fixation can become evident between 8 to 15 days following inoculation (these are usually large pink or red nodules) with their colour resulting from the presence of leghaemoglobin which protects nitrogenase from O_2 (FAO, 1984). The presence of leghaemoglobin is a unique characteristic between rhizobia and leguminous plants (Postage, 1971), the plant produces this heme protein which has a strong binding affinity for O_2 removing O_2 toxicity to the nitrogenase complex and delivering O_2 to the bacteria electron-transfer system (Nelson and Cox, 2013). A nodule that becomes active may only be capable of N_2-fixation for 50 to 60 days, therefore, nodule formation on plants in a field may have more than one flush in a growing season. However, it should be noted that only a small proportion of infections result in nodule formation (Sylvia et al., 2005).

1.4. Significance of BNF in grassland agriculture

The Grass (Poaceae formely Gramineae) family comprises over 620 genera and 10,000 species, which are spread throughout the world, with typical agricultural species including *Poa*, *Festuca*, *Lolium* and *Dactylis* (Haynes, 1980; Campbell, 1985; Christenhusz and Byng, 2016). In the UK, grasslands cover a significant part of the landscape, in fact in England alone over half of the agricultural land is covered by grasslands with much higher percentages in Scotland and Wales (Fowell, 2010). Europe was one of the first places in the world to recognise the problem of over use of synthetic fertilisers, seeing decreased application in the 1990s, however, use is steadily increasing again (Erisman et al., 2011). Despite the decreasing trend and the application rate remaining consistently lower than to tillage crops, grasslands still represent a major source of industrial fertiliser input, receiving 56 kg ha^{-1} alone in 2016 (Figure 1.10). It has been suggested that it is both possible and desirable to reduce the use of synthetic N fertilisers and to move towards a greater use of legumes capable of BNF (Crews and Peoples, 2004; 2005), where N can be transferred to an associated crop (Jensen, 1996a, b; Mahieu et al., 2007).

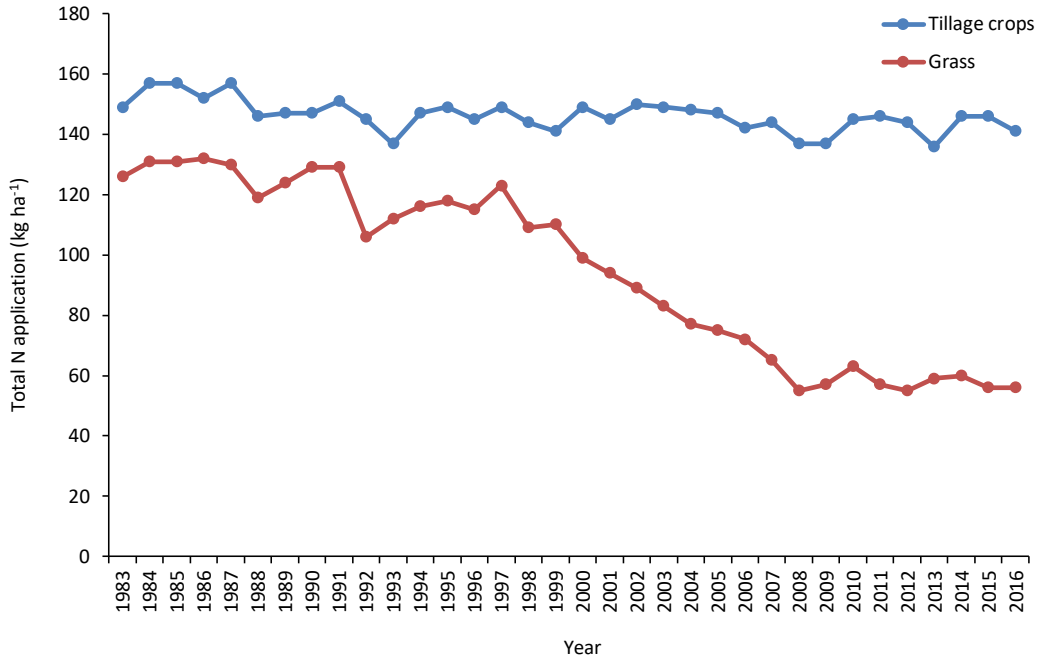


Figure 1.10. Overall nitrogen application rates (kg ha^{-1}) in Great Britain between 1983 and 2016 for tillage crops and grassland (adapted from Department for Environment, Food and Rural Affairs [Defra], 2017, the British Survey of Fertiliser Practice).

There has been renewed interest in the use of clover (*Trifolium spp*) within grasslands, due to the capability of clover to fix atmospheric N allowing reduced N fertiliser application on grasslands bringing economic and environmental benefits (Boller and Nosberger, 1987; Schils, 2002). In temperate zones of the world, white clover (*Trifolium repens* L.) is often recognised as the most important pasture legume of agronomic significance (Frame and Newbould, 1986; Brock et al., 1989; Elgersma et al., 2000; Kušliene et al., 2014), especially within low-input or less intensively managed agricultural systems (Dahlin and Stenberg, 2010a). Furthermore, within pasture production a critical factor is the interaction between legumes and associated grasses (Haynes, 1980). To benefit from BNF, two main agricultural practices are often used, either with the use of crop rotations or intercropping (Fustec et al., 2010). However, mixed cropping of clover and grass swards has been found to stimulate N_2-fixation (Barea et al., 1989a), with white clover being shown to derive up to 75% of its N needs from N_2-fixation (Boller and Nosberger, 1987; Barea et al., 1989b). Furthermore, out of all legumes, white clover has been found to be the most generous in contributing excess N to associated plant species (Pirhofer-Walzl et al., 2012; Rasmussen et al., 2012) with as much as 50% of N in grasses being found to be orginated from the legume (Soussana and Hartwig, 1996; Høgh-Jensen and Schjoerring, 2000; Gylfadóttir et al., 2007; Rasmussen et al., 2007; Rasmussen et al., 2013). Growing clover and grasses in mixed swards provides a wide range of additional benefits, such as; increased total yield of both crops, helping to further improve soil fertility, enhanced nutrient status, improved efficiency of water use, providing weed control as well as providing protein-rich high quality forage for sheep and cattle (Haynes, 1980; Ta and Faris, 1987; Macduff et al., 2002). Mixed swards tend to overcome the problems of growing white clover in monocultures, which include problems with; keeping the sward weed free, low annual herbage production, short growing seasons and concern over bloat and reduced reproduction in grazing livestock (Frame and Newbould, 1986). The latter point is of particular importance, as within the UK nearly all grassland is grazed at some stage during the year, for instance, in 2016 this use represented 92% of all grassland (DEFRA, 2017).

Despite the white clover and grass relationships having been intensively studied, two major restrictions have limited their use:

(i) the lack of understanding and matching of the amount of N made available by the legume crop to that of the demand of the non-legume, and

(ii) the lack of understanding of the N-transfer pathway for N through the soil from the N-fixing legume to the associated non-legume.

The concept of synchrony was originally proposed by Swift (1985) and can be summarised as "the release of nutrients from aboveground inputs and roots can be synchronised with plant growth demands", ultimately this is the relationship of two or more events occurring at the same time (Bath, 2000). Synchronisation typically refers to nutrients from biological cycling: N, P and S, for example, and the availability of mineralised N at times of crop demand. Alternatively, if the events are not simultaneous, asynchronisation occurs, this is often the source of environmental problems associated with excess N in agriculture (Crews and Peoples, 2005). Asynchrony may occur for two reasons: (i) when nutrient availability exceeds plant requirements due to nutrients being added or released at a time when plant demand is limited or absent; such as in the winter or early spring in the UK, also known as excess-asynchrony, or (ii) when nutrient supply is insufficient to meet plant demands, such as nutrients being released at a rate slower than the plant's needs, also known as insufficient-asynchrony (Myers et al. 1994). Furthermore, in a cropping system where legumes are providing the main source of N, synchronisation also depends on: chemical and morphological composition of the crop effecting decomposition (determining mineralisation), spatial distribution of the plant material and the soil type allowing transfer of nutrients (Bath, 2000).

It still remains unanswered whether a fertiliser- or legume-based system has a greater potential for attaining synchrony. Studies which have looked at the synchronisation in synthetic fertiliser and legume-based systems typically show rainfed agriculture crops to recover more N from fertilisers than from legume derived N. However, in legume-based systems more N is retained in the soil (Ladd and Amato, 1986; Janzen et al., 1990; Harris et al., 1994; Bergström and Kirchmann, 2004; Crews and Peoples, 2005). However, legume-based systems could better achieve synchrony than synthetic fertiliser-based systems if strategies were used to address decreasing the periods of excess nutrient availability. Typically, asynchrony commonly occurs after fertilisation in the early growing season, this is when soil available N far exceeds the crops demand. Whereas, the incorporation of clover residues has been shown to remain either immobilised or undecomposed in the soil, and hence provides a more gradual release of N to the crop than fertilisers (Groffman et al., 1987).

It is often assumed that the growth of mixed clover and grass is straightforward, with two main processes governing their success: temperature and moisture, which control N release and uptake through decomposition, net primary productivity (NPP) and plant nutrient demand (Rosenwig, 1968; Myers et al., 1994). However, clover and grass have opposing responses to

soil mineral N (Griffith et al., 2000), it is typically found that clover cannot supply enough N through N_2-fixation for high herbage grass yields throughout the growing season, therefore, N fertilisers are applied to supplement N (Clement and Jones, 1977). For example, it is estimated that clover can fix 80-100 kg N ha^{-1} yr^{-1} (Ledgard et al., 1996; Ledgard, 2001), which is currently above fertiliser application rates to grasslands (Figure 1.10) but would require all N to be available to the associated crop along with no losses to the environment. Following the supply and uptake of soil mineral N in clover, N_2 fixation is down-regulated, which is advantageous to clover as it can compete for soil N (Chapman et al., 1996) thus reducing the high metabolic cost of N_2-fixation (Ryle et al., 1979) (Figure 1.11). Frequent applications of industrial fertilisers is a practice which needs to be minimised due to the economic and environmental costs associated, but also this decreases the overall clover content present in the sward due to increased competition from grass (Macduff et al., 2002). Although results for long-term studies are variable (Clement and Jones, 1977; Griffith et al., 2000), and short-term experiments have shown that clover growth rates are independent of mineral N availability (Griffith et al., 2000). Research has shown that a 'strategic' approach where only a spring application of N is applied can be effective on grass-clover swards, increasing dry matter yield in the first cut with only a temporary reduction in clover content (Schils, 2002).

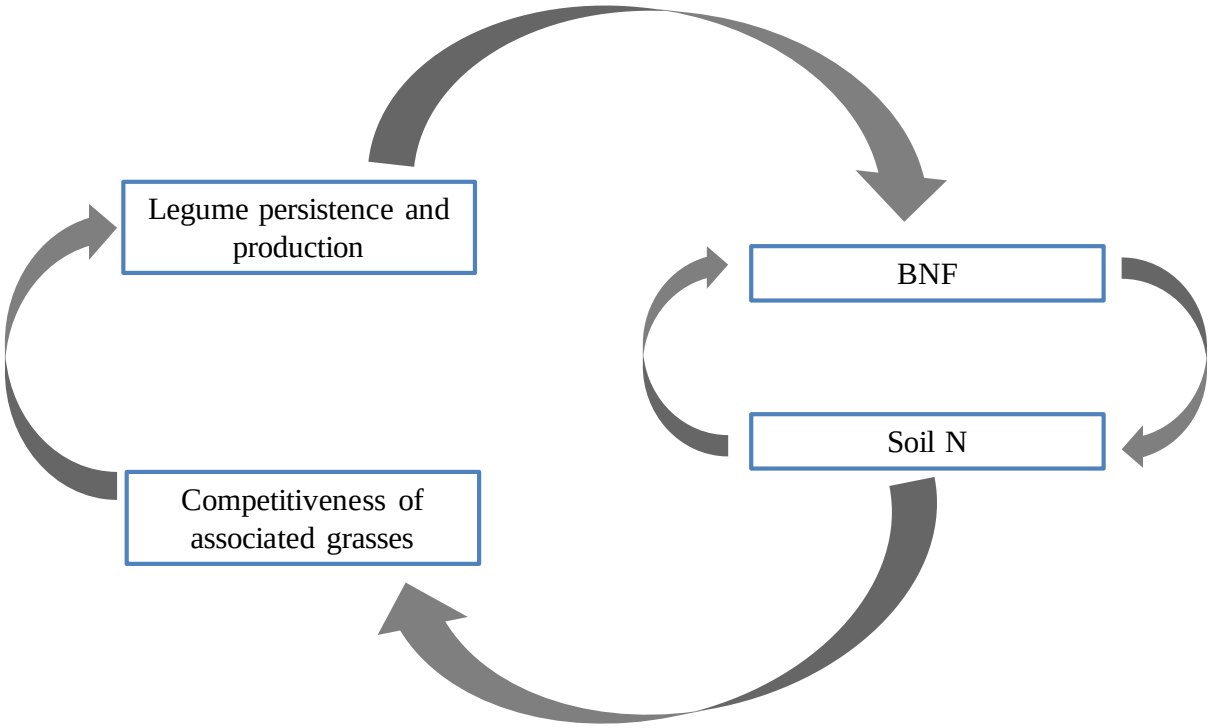


Figure 1.11. Major factors which interact to determine BNF, showing the trade-off between soil N and BNF. (Adapted from Ledgard and Steel, 1992).

There is much conflicting evidence as to how the associated non-legume crop obtains the N which can be made available by the N_2-fixing legume crop as well as a lack of evidence about relevance, quantitative importance and controlling factors of each transfer pathway, due to technical difficulties in investigating belowground processes (Gylfadóttir et al., 2007). This is the area of legume research where increased knowledge is needed to make intercropping systems more effective (Stern, 1993). It is found that the different pathways and the amount of N actually transferred are dependent on growing conditions (e.g. soil and climate), species used, year, sward age and management practice (Whitehead, 1970; Goodman and Collison, 1986; Wacquant et al., 1989; Murray and Clements, 1998; Ramussen et al., 2013; Louarn et al., 2015), meaning that the dominant processes are likely to be highly variable. The transfer pathways had even previously been regarded as a 'black-box' (Ross et al., 1972) and are important to identify and quantify as N-transfer determines the success of an intercropped system (Stern, 1993). Commonly, studies tend to classify only three major routes for transfer of N: plant exudation, decomposition (root and nodule sloughing off, senescence and decay) or *via* mycorrhizal fungi interconnecting the roots of different plant species (Virtanen et al., 1937; Ta et al., 1986; Wacquant et al., 1989; Bethlenfalvay et al., 1991; Murray and Clements, 1998; Paynel and Cliquet, 2003; Rasmussen et al., 2013; Thilakarathna et al., 2016) (Figure 1.12). Previous studies have suggested that direct N-transfer from a legume to a non-legume might not take place under all soil conditions or, alternatively, it may only occur gradually (i.e. mineralisation) (Peoples and Craswell, 1992). Further to this, it is typically thought that nodule and root decomposition is the principal N-transfer pathway, involving complex cycling of organic material (Goodman, 1988; Haystead and Marriott, 1979; Ta and Faris, 1987; Dubach and Russelle, 1994; Trannin et al., 2000; Sierra et al., 2007). Furthermore, studies have concluded that N-transfer between plants cannot be completely explained by the competition for inorganic N, showing that organic N has an underlying part within transfer (Rasmussen et al., 2013). Underpinning the contribution of these different mechanisms is a key aim within this thesis, these different processes are further studied in more detail within Chapters 5 and 6, as well as the possible contributions of aboveground processes and plant herbivores to N-transfer, leading to a better understanding of the factors which regulate N cycling.

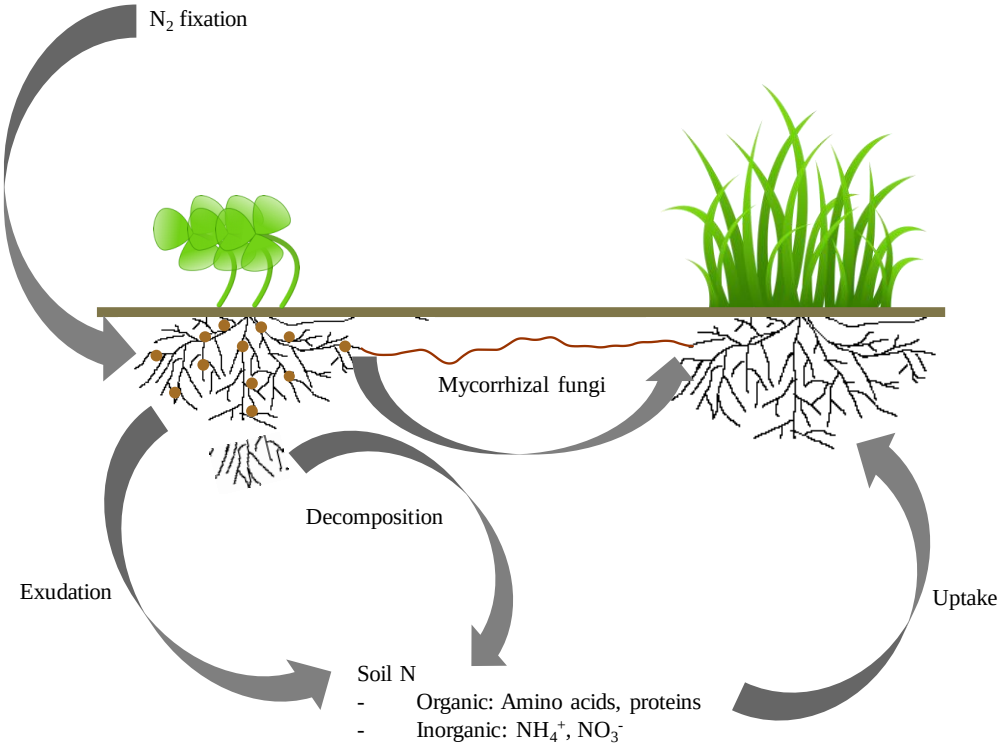


Figure 1.12. Commonly classified major routes of N-transfer between a N_2-fixing legume and a non-legume plant species.

1.5. Approach and aims

The global need to reduce industrial fertiliser use is clear as is a move towards more sustainable forms of N through the inclusion of BNF legumes either by intercropping or crop rotations, however, to do so a better understanding of N cycling and N-transfer from a legume to a non-legume is needed. To assist in the study of the fate and flow of N through plant and soil systems, stable N isotopic labelling can be employed allowing N to be traced, furthermore, this can be used at a variety of scales from greenhouse experiments to landscapes (Bedard-Haughn et al., 2003). This can be achieved due to the fact that N exists in two stable isotopes, where 99.6337% of the atmosphere is composed of ^{14}N with the remainder as ^{15}N (Leinweber et al., 2013). To ensure that the isotopic signature of N can be found, substances may be artificially enriched with ^{15}N ('^{15}N-labelled') guaranteeing that there is a meaningful difference between the source and background levels of ^{15}N, further allowing the detection and quantification of inputs or losses of N. Artificially, ^{15}N enriching substances can be achieved in a number of different ways, such as through fertiliser application to plants, which when applied becomes part of the overall N cycle which has a unique isotopic signature (Bedard-Haughn et al., 2003). This technique has been used with agronomic research for more than 80 years (Hauck and Bremner, 1976), and is accepted as the most reliable way to determine the flow and fate of N (Bedard-

Haughn et al., 2003). More recently techniques have been developed for investigating the N input from living roots into bulk soil (rhizodeposition) to better understand their contribution to total N budgets (Schmidtke, 2005; Wichern et al., 2008; Fustec et al., 2010). This can be expanded further to determine their fate within different soil N pools (Schweinsberg-Mickan et al., 2010), such as into the organic soil N pool where 95% of soil N remains (Bedard-Haughn et al., 2003). To date, very few studies have investigated the chemical composition of organic N compounds released from plants (Hertenberger and Wanek, 2004; Merbach et al., 1999) and typically studies look at the bulk ^{15}N enrichment of soil. One method that can be used to determine the biomolecular fate of N released by plants into the soil organic N pool is the use of compound-specific ^{15}N stable isotope techniques, such as within AAs, providing valuable insights into the active cycling of N and N turnover processes in soils as well as potential microbial transformations.

As identified in Section 1.4, a lack of understanding regarding the pathway of N-transfer from clover-to-ryegrass has limited their use and closing this knowledge gap would be extremely valuable in improving NUE and using more sustainable N sources. The work presented in this thesis, therefore, focuses on using stable ^{15}N isotope techniques at the bulk level, complimented with compound-specific N isotope approach. This will enable further investigation at the molecular level to further elucidate the mechanisms, N turnover processes and N-transfer pathways responsible for the release of N from clover and, subsequently, how this meets the N demand within ryegrass. The specific objectives relating to this work are to:

- Develop a robust method to introduce ^{15}N compounds to clover plants which will allow assessment of N-transfer from clover-to-ryegrass, access the microbial assimilation and biomolecular fate of N in soils.
- Determine whether a sustainable source of N can be supplied from clover to ryegrass.
- Investigate the routing and controls of N from clover-to-ryegrass using ^{15}N isotope labelling techniques.
- Determine the role of soil biota in N-transfer from clover-to-grass.
- Develop new land-use management strategies for the sustainable transfer of N from clover-to-ryegrass.

Chapter 3 provides a short review of methodologies which have been previously developed for introducing a ^{15}N-label into an already established plant system. Laboratory experiments are used to compare commonly used techniques of introducing ^{15}N into a single plant species.

These findings are expanded upon in Chapter 4, where methods and expressions for calculating the amount of N-transferred between plants are compared. The initial two chapters look at advancing established techniques to provide a robust method which is appropriate for addressing the aims of this thesis. The development is then taken forward to Chapter 5, where the different mechanisms for N-transfer between plants is further reviewed and investigated, addressing the hypothesis- H1. Chapter 6 further uses the developed method to investigate the role of soil biota in N-transfer and whether N-transfer can be further enhanced through manipulating the biota community, addressing the hypothesis- H2. The two major hypotheses that will be investigated in this thesis are:

- H1- It is hypothesised that decomposition will play a larger role in ^{15}N uptake in ryegrass and therefore N-transfer from clover-to-ryegrass than exudation. Removing the clover shoots by cutting will accelerate death and decomposition substantially increasing the ^{15}N uptake in ryegrass and therefore N-transfer from clover-to-ryegrass.
- H2- It is hypothesised that soil biota plays a vital role in mediating the transfer of N originating from clover-to-ryegrass, elimination of soil biota will significantly reduce N transfer, while enhancement of certain soil biota will enhance N-transfer.

Chapter 2

Materials and methods

2. Materials and methods

2.1. Overview

The overall aim of this thesis is to trace the N flow from clover-to-grass plants using ^{15}N-labelling techniques in order to get a better understanding of the routing and controls of N-transfer. This chapter focuses on the methods used throughout this thesis to address the overall aim. Firstly, the soils which were sampled are described as well as establishing a clone mother clover and ryegrass plants which have been maintained and grown throughout the duration of the experimental work. The general approach to setting up experiments using both rhizotrons and incubation tubes for Chapters 3-6 are described, however, the specifics of each experiment (such as growth periods) are described within individual chapters. This chapter also describes the experimental protocols, instrument analysis and equations used throughout this work. Where experimental techniques are only applied in one particular experiment, details are provided in the relevant chapter.

2.2. Sample collection, and growth medium preparation- soil and sand

2.2.1. Site description

Soil samples were collected from the North Wyke Farm Platform (NWFP), located near Okehampton, Devon, South-west England (50°46'N, 3°54'W), which is part of Rothamsted Research (Figure 2.1). North Wyke is a permanent grassland system. The underlying geology is the Carboniferous Crackington Formation consisting of clay shales with thin sandstone bands. The soils on the farm platform are poorly drained with clay throughout and an impermeable clay layer at 30cm (Harrod and Hogan, 2008). The soils are of non-calcareous Pelo-stagnogley of the Hallsworth series forming a typical greyish coloured soil and non-calcareous pelosol of the Halstow series forming a brownish coloured soil (Avery, 1980; Harrod and Hogan, 2008). The mean annual rainfall is 1055.7 mm and a mean annual temperature of 9.6ºC (mean values over the 40 year period 1961-2000) (Harrod and Hogan, 2008). The soil is a slightly stony clay loam topsoil (~36% clay) overlying a mottled stony clay (~60% clay) (Murray et al., 2013; Orr et al., 2016).

NWFP is managed as a conventional intensive sheep and beef production system, whose vegetation reflects the typical land-use system in the southwest of England with predominately

Lolium spp. interspersed with *Cynosurus, Festuca, Agrostis, Holcus and Dactylis* spp (Bol et al., 2004; Peukert et al., 2012). In 2010, the farm platform was established, dividing the land into three individual farmlets, each of approximately of 21 hectares, with beef and cattle production taking place on the land. However, the transition phase did not begin until April 2013, where the different treatments of the three farmlets were established:

- Permanent pasture (control): continued sward improvement of the existing grassland using artificial fertilisers which consists of mainly perennial ryegrass.
- Increased use of legumes: re-seeding with long-term grass (perennial ryegrass*; Lolium perenne* L.) and legume (white clover; *Trifolium repens* L.) mixtures. However, the N source is not completely reliant on clover, with a maximum of 40 kg N ha^{-1} of inorganic fertiliser application particularly in cold slow growing seasons, with additional use of organic manure.
- Planned reseeding: with reseeding roughly every four years, trialling new varieties of grass (improvement through innovation), such as those with improved animal performance (e.g. enhanced sugar content) or environmental resilience (e.g. deep-root grasses) (Murray et al., 2013; Orr et al., 2016).

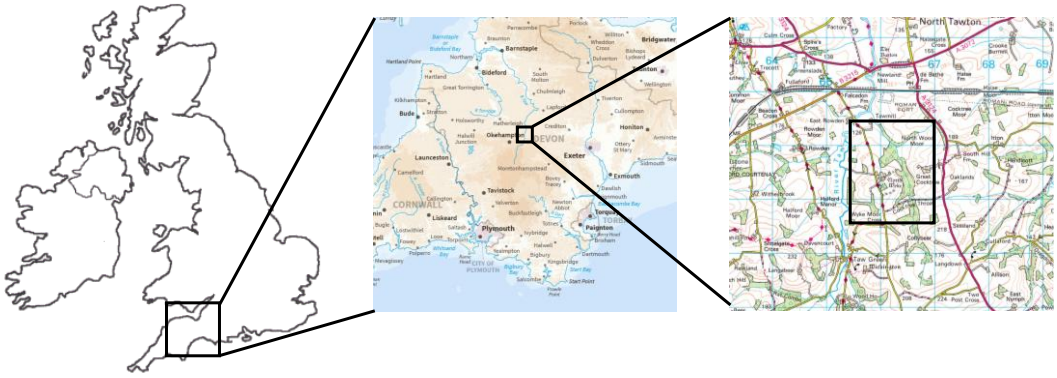


Figure 2.1. Map showing the location of the North Wyke Farm Platform. © Crown Copyright and Database Right [06/02/2018]. Ordnance Survey (Digimap Licence).

2.2.2. Soil collection

Between June and July 2012, a total of 264 samples were collected from NWFP. A 50 m grid in ArcGIS shows where the geo-referenced grid points were located using a handheld Trimble GPS unit (Figure 2.2). Soil samples were collected by pressing a cylindrical core with 55 mm diameter and 100 mm height into the soil. Samples were then bagged and stored at +4˚C until required.

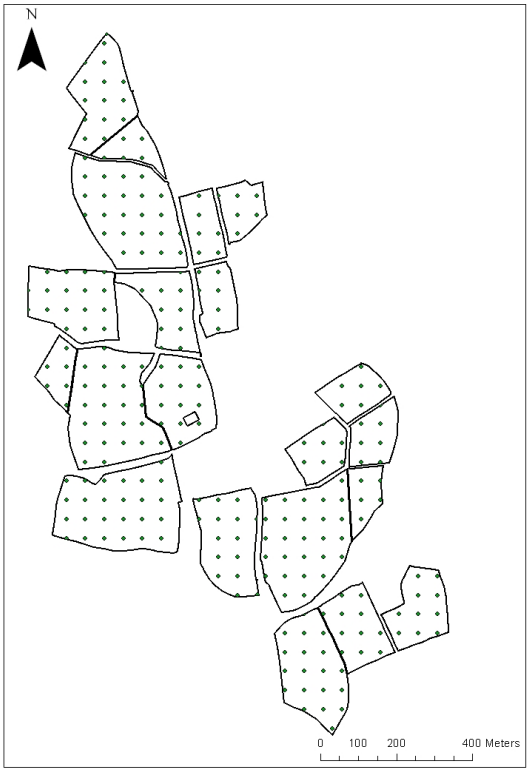


Figure 2.2. Location of the fields within the North Wyke Farm Platform shown in Figure 2.1, green dots represent the location of the soil sampling sites.

2.2.3. Soil storage and preparation

Soil cores were broken up and placed out to air-dry before being sieved to 2 mm. After sieving, tweezers were used to remove any large pieces of root material which had passed through the sieve. Soils were mixed together, then bagged and stored at +4˚C until required, which allowed any disturbance to the microbial community to be minimised.

All experiments conducted in chapters 3-6 used the same soil growth medium described here. The soil had a total carbon content (%TC) of 5.23%, and a total nitrogen content (%TN) of 0.57%. The pH of the soil was 6.3.

2.2.4. Sand preparation and storage

A horticultural silver sand (Vitax) was washed five times in distilled water to help remove fine particles before being acid washed with 0.5 M HCl by soaking the sand in the acid overnight twice. The sand was then washed with distilled water until the pH of the standing water returned to neutral as confirmed with pH paper. The sand was then oven dried at 75°C before being sieved to 1 mm, the sand was then furnaced at 450°C overnight. The sand was stored in sealed furnaced glass jars at room temperature until required.

2.2.5. Clover mother plant

To reduce the potential effects on the results arising from genetic variation a clover mother plant was grown (established February 2014). A soil-based compost (John Innes number 3) was used to fill two pots and then compacted. Five seeds of white clover (*Trifolium repens*) cv. Aber Dai were placed into each pot then covered with further soil to a depth of 0.5 cm and further compacted. Pots were watered and then placed in sunbags (Sigma-Aldrich, Dorset, UK) at room temperature and allowed to germinate. Clover plants were allowed to grow for six weeks before being separated and planted into individual pots. After initial further growth period of two months one clover plant was chosen at random to go be the mother plant for all future experiments, this was then re-potted into a large pot. Stolon produced by the mother plant were placed over trays of damp compost to encourage the growth of adventitious roots (Figure 2.3), these were then cut and re-potted producing a genetic clone of the mother plant. Clover plants were watered with tap water every day and watered with a modified Hewit solution omitting N once a week (Section 2.2.7).

For the majority of the project, the mother clover plant was grown in the Bristol experimental greenhouses, under fully computer controlled conditions for heating, supplementary lighting, ventilation and shading. During the initial few months when the mother plant was being established, the plants were grown at North Wyke Rothamsted Research. The day and night temperatures of the greenhouse were set to 20.0°C, however, due to external factors the maximum and minimum temperatures fluctuate, these can be found in Table 2.1. All plants had a 16 h photoperiod from 5 am to 9 pm, supplementary lighting was provided through 150 W 50/60 Hz High output, Correct Spectrum Class 11 energy saving bulbs. Supplementary lighting was provided when natural daylight levels were below 30 W/m2, and turned off when natural

light levels exceeded 50 W/m^2. Additional shading was provided when natural light levels exceeded 220 W/m^2 and removed when levels dropped to 100 W/m^2.

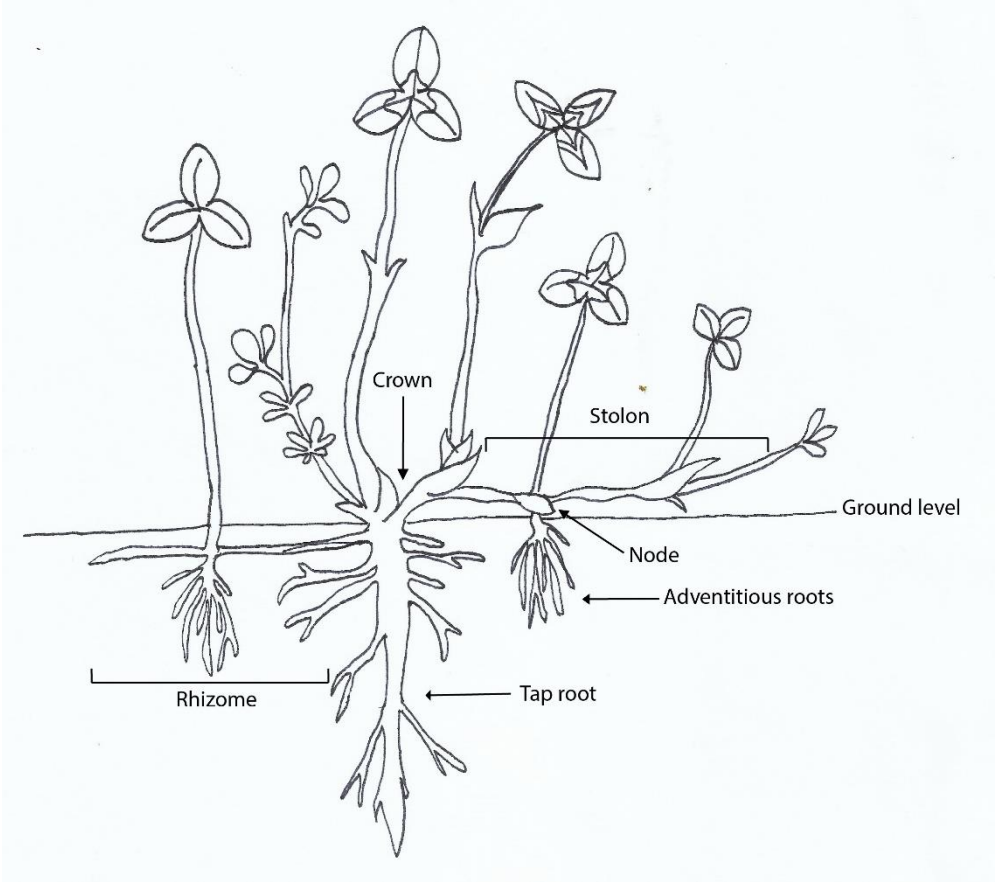


Figure 2.3. Anatomy of clover, showing the above and below ground parts, and the stolon with adventitious roots which can be cut and re-planted to produce clone plants. (Diagram provided by Ellie Britton, with permission.)

Table 2.1. Maximum, minimum and average temperatures (°C) in the greenhouse throughout the duration of the project.

	Max	**Min**	**Avg**		**Max**	**Min**	**Avg**
Aug-14	35.4	17.6	22.1	Apr-16	33.1	17.6	20.5
Sep-14	32.1	17.6	22.2	May-16	35.7	17.6	22.4
Oct-14	28.6	16.7	20.5	Jun-16	40.2	17.8	22.2
Nov-14	24.7	16.7	20.0	Jul-16	40.8	17.6	23.4
Dec-14	22.9	16.3	19.8	Aug-16	38.7	16.5	23.2
Jan-15	23.7	16.8	19.6	Sept-16	36.4	17.8	22.4
Feb-15	23.4	16.2	20.0	Oct-16	33.0	17.8	21.0
Mar-15	27.0	16.7	20.1	Nov-16	24.2	18.4	20.1
Apr-15	33.8	17.3	21.6	Dec-16	23.2	18.4	20.1
May-15	43.1	17.4	21.3	Jan-17	23.3	18.5	20.3
Jun-15	40.6	17.6	23.1	Feb-17	24.6	18.5	20.0
Jul-15	38.8	17.6	22.8	Mar-17	29.4	18.1	20.5
Aug-15	37.5	17.6	22.3	Apr-17	35.4	18.2	21.6
Sept-15	35.9	16.8	22.0	May-17	40.4	19.3	22.8
Oct-15	28.0	16.8	20.3	Jun-17	44.6	19.3	23.4
Nov-15	26.8	17.1	19.8	Jul-17	41.3	19.6	24.3
*Dec-15	21.8	15.7	17.9	Aug-17	38.5	19.5	23.1
Jan-16	21.5	15.5	17.8	Sept-17	34.5	19.1	21.9
Feb-16	23.4	15.5	18.0	Oct-17	29.9	18.6	21.0
Mar-16	29.2	15.5	19.9				

* Indicates when plants were moved to a different compartment of the experimental greenhouses, from the "mycology" section to the "end" section of the greenhouse.

2.2.6. Ryegrass mother plant

Similarly to the clover mother plant, a ryegrass mother plant was produced to eliminate effects on the results arising from genetic variation (established June 2014). A soil based compost (John Innes number 3) was used to fill two pots and compacted. Ten seeds of ryegrass (*Lolium perenne*) cv. Aber Magic were placed into each pot which was then covered with further soil to a depth of 0.5 cm and compacted further. Pots were watered and then placed in sunbags (Sigma-Aldrich) at room temperature and allowed to germinate. Ryegrass plants were allowed to grow for 6 weeks before one ryegrass plant was chosen at random to be the mother plant. Once the plant had produced 8 stolon (Figure 2.4), these were then divided to include as much root as possible and each was planted into a separate pot of compost. Ryegrass plants were then allowed to grow again to produce roughly 8 stolon before being divided again. The process of dividing the ryegrass plants continued until sufficient were generated to conduct an experiment. Ryegrass plants were watered every day and watered weekly with the modified Hewit solution,

this time included the application of N (Section 2.2.7). The mother ryegrass plant was grown in the experimental greenhouses alongside the clover mother plants throughout the duration of the project, the maximum, minimum and average temperatures are shown in Table 2.1.

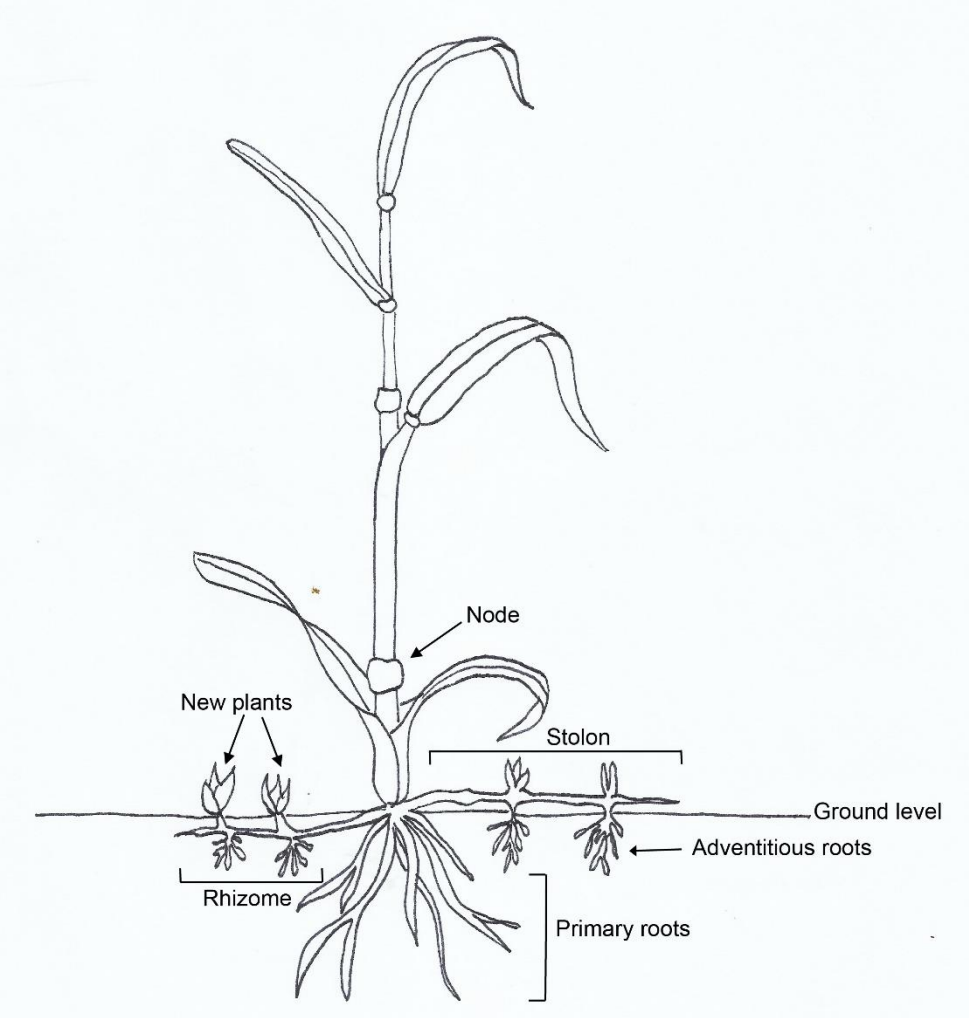


Figure 2.4. Anatomy of ryegrass, showing the above and below ground parts, and the stolon with adventitious roots which can be cut and re-planted to produce clone plants. (Diagram provided by Ellie Britton, with permission.)

2.2.7. Nutrient solution- Modified Hewitt solution

Nutrient solution (1 L) was prepared using the modified version of Arnon's solution at 1/5 of the strength (Hewitt, 1966) for the weekly watering of mother plants (Sections 2.2.5 and 2.2.6) with the remainder being made up from DDW. The modified Arnon's solution allows for N to be omitted; this was used for the mother clover plants to encourage nodulation. Where plants were replanted for the running of experiments (in rhizotrons or incubation tubes), full strength Arnon's solution was used with N omitted (apart from where detailed in individual experiments). Stock solutions of all the components in Table 2.2 were produced before being mixed together within the nutrient solution.

Table 2.2. Component and volume required of the modified Arnon's solution to make 1 L of nutrient solution.

		Mass (g) to make up 1 L of stock solution	Required volume (mL) of stock solution for making 1 L of nutrient solution	Required volume (mL) of stock solution for making 1 L of nutrient solution at 1/5 strength
Macro-nutrients	NH_4NO_3	11.43	50	10
	K_2SO_4	6.69	66.7	13.34
	$Ca(H_2PO_4)_2{\cdot}4H_20$	2.52	50	10
	$MgSO_4.7H_2O$	9.86	50	10
Trace elements	H_3BO_3	2.86	1	0.2
	$MnCL_2{\cdot}4H_2O$	1.81	1	0.2
	$CuSO_4{\cdot}5H_2O$	0.08	1	0.2
	$ZnSO_4{\cdot}7H_2O$	0.22	1	0.2
	H_2MoO_4	0.09	1	0.2
Iron	$FeSO_4{\cdot}7H_2O$	7.47	1	0.2
	Conc. H_2SO_4	0.25 mL	1	0.2

2.2.8. Glassware and other experimental equipment

All experimental equipment was washed with Decon 90 (Decon Laboratories Limited, East Sussex, UK), and rinsed with water. Glassware (rhizotron glass plates and incubation tubes) were then rinsed with acetone and oven dried before being furnaced at 450°C for 3-4 hours. Plastic rhizotron backs (Figure 2.5) were further rinsed with DDW (double distilled water, produced by a Bibby Aquatron DDW still) before being oven dried (any residual solvent would have attacked the plastic and melted the glue holding the plastic sides together). Glass wool was also furnaced at 450°C for 3-4 hours. All volumetric glassware and pestles and mortars

(unsuitable for furnacing) were solvent rinsed with dichloromethane (DCM) following Decon and acetone washing. Any DDW which was not being applied to living plants or soil (i.e. for the amino acid protocol) was further purified by extracting with DCM in order to remove any remaining organic contaminants which could not be removed through the distillation process.

2.2.9. Rhizotrons

Rhizotrons were created from a hard plastic plate (internal dimensions of 11 by 23.5 cm), and plastic strips of 1 cm width were stuck onto the perimeter to give a soil profile of 0.5 cm in depth (Figure 2.5). A gap (~1 cm) was left at the bottom of each rhizotron and filled with furnaced glass wool to allow water flow and prevent soil falling out. Each rhizotron was filled with 100 g of prepared air-dried soil (Section 2.2.3) and levelled before a glass plate covering the whole rhizotron structure was placed on top to hold the soil in place (13 by 24 cm). Each rhizotron was then wrapped in tin foil to keep the root profile in the dark and held together with three bulldog clips.

For the rhizotrons, clover and/or grass plant cuttings were taken from the mother plant by removing stolon with adventitious roots, these were rinsed with DDW. Plants were introduced into the rhizotrons by replanting on the top of the rhizotron soil surface with any additional root matter being introduced into the soil profile. The soils were then re-wetted to approximately 60% water holding capacity (WHC).

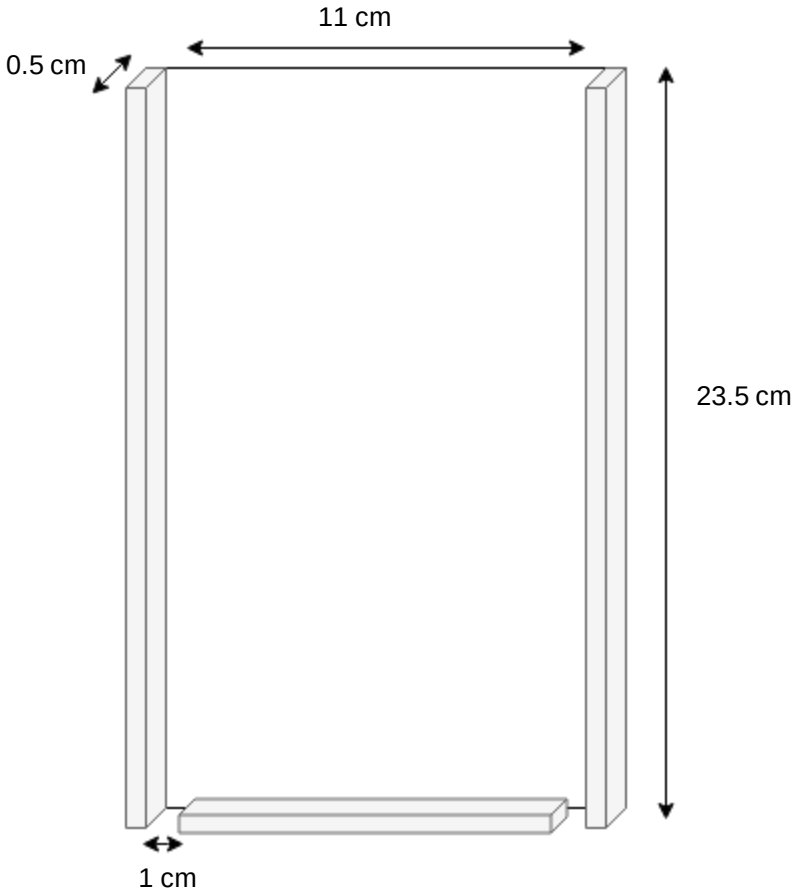


Figure 2.5. Rhizotron plastic backs used for the growing of clover and/or grass in experiments, where a glass front of 13 cm × 24 cm was placed over the top, wrapped in foil and held on by bulldog clips.

For the introduction of a ^{15}N-label to plants in rhizotrons, the labelling solution was either injected into the soil (which is described in individual experiments using this technique). Alternatively, ^{15}N-label was introduced *via* a leaf-labelling technique, which involved submerging leaves in the solution, similar to the method described by Ledgard et al. (1985) and Murray and Hatch (1994). Where, two attached leaves from each clover plant were immersed in the ^{15}N enriched solution in 7 mL glass vials, which were taped onto the front of the rhizotrons and covered with Parafilm (Sigma-Aldrich) to reduce evaporation of the solution. Care was taken to avoid any contact with the rest of the plant, after 72 hours the immersed leaves were cut and removed with the vials.

2.2.10. Split-root technique in incubation tubes

Experimental cloned ryegrass and clover plants were grown in small soil incubation tubes as described by Knowles (2009), Knowles et al. (2010), Charteris et al. (2016) and Charteris (2016). These consist of open topped glass tubes with a height of 10 cm by a diameter of 2 cm (Figure 2.6), with a tapered small hole plugged with furnaced glass wool at the base allowing drainage to prevent water-logging, and stop soil from falling out.

Three weeks prior to the setting up of incubation tubes for each experiment, a corresponding number of additional clones of the mother clover and ryegrass plants were taken for the running of each individual experiment (Section 2.2.5 and 2.2.6) by removing stolon with adventitious roots. Clover and ryegrass plants were re-potted into plastic plant pots (9 cm in diameter and 9 cm tall) in compost (John Innes number 3) to allow substantial root growth for the experiment (at least 5.5 cm in root depth). After three-weeks plants were removed from pots and excess compost removed by washing with DDW, plant roots were divided roughly in half and fed down each side of glass Y-tubes (5 cm high x 0.8 cm diameter) (one plant per Y-tube). The Y-tubes were used to hold and separate the plant roots between different incubation tubes as well as preventing the desiccation of plant roots. Plant roots were placed into incubation tubes with prepared glass wool at the base. Sieved, prepared air-dried soil used for the transfer (TC) and receiving compartments (RC) (15 g, Section 2.2.3) and furnaced sand used for the labelling compartment (LC) (25 g, Section 2.2.4) was weighed out into 28 mL glass vials before being slowly poured around plant roots in the incubation tubes and tamped down. Incubation tubes were then brought up to approximately 60% WHC using DDW. Incubation tubes were initially sat in a small glass vial which was filled with DDW to help the re-wetting of the soil and to help plants adapt to new growth conditions. After the initial growth period (see individual chapters for time details) these were exchanged for clean empty vials to catch any water run-off during the experimental period. Incubation tubes were wrapped in silver foil, to keep the roots in the dark, and held in test tube racks.

All incubation tubes and plants had an initial growth period of at least three weeks to allow plants to acclimatise and start new growth (although this varied slightly from experiment to experiment depending on plant growth, (see details in individual chapters). Substrates DDW or 30 mM $^{15}NH_4{}^{15}NO_3$ at 98 atom % or 30 mM $CO(^{15}NH_2)_2$ at 98 atom % were introduced by injection into the sand (LC) where the needle was drawn up as the plunger was pressed (as previously conducted by Charteris, 2016; Charteris et al., 2016). Each LC received four 0.25

mL or five 0.2 mL injections spread out over the sand profile in order to achieve maximum distribution (Murphy et al., 1999). After introduction of the substrates the timed experiment commenced with sampling at either 100 h or 480 h. All incubation tubes were watered with a full strength Arnon's solution daily (Section 2.2.7).

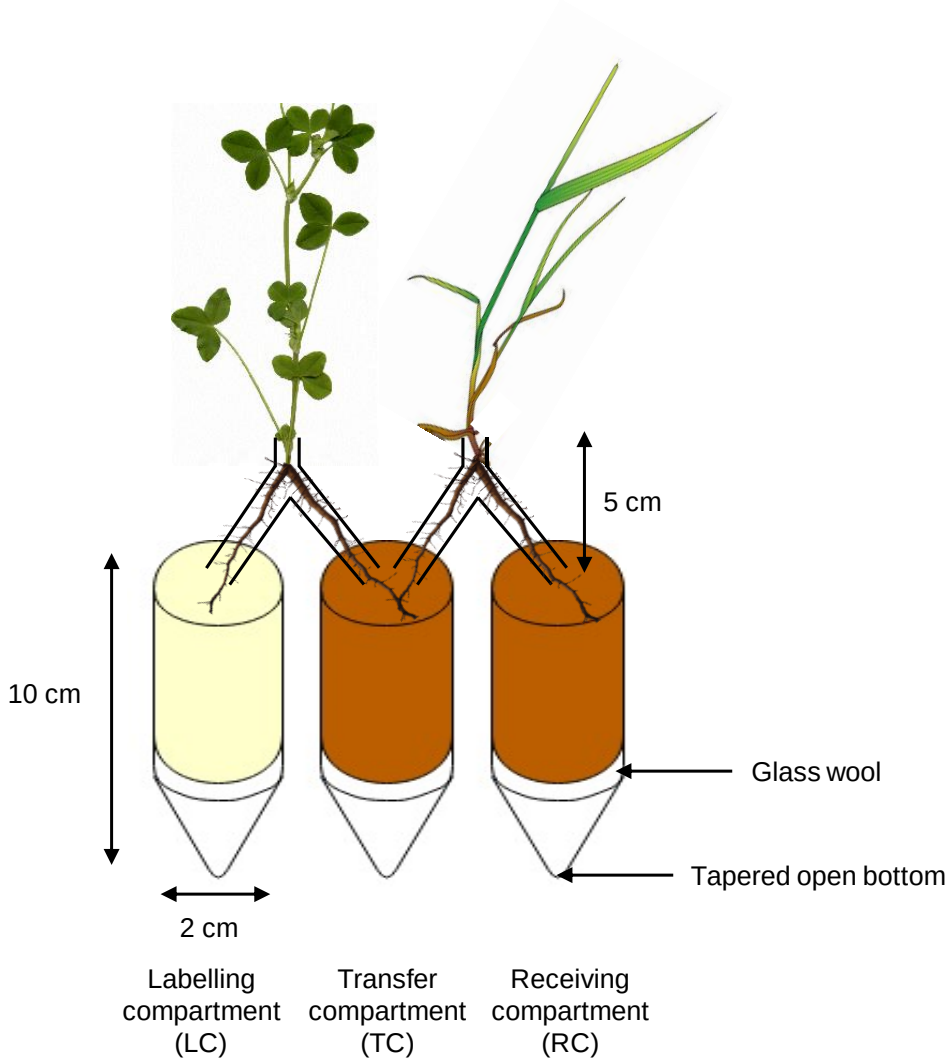


Figure 2.6. Incubation tube set up, with sand filled labelling compartment (LC), soil filled transfer and receiving compartments (TC and RC, respectively) with a glass wool plug, and glass Y tubes supporting the roots between the different compartments.

2.3. Bulk C and N analysis

All samples were analysed for total C, N and $\delta^{13}C$, $\delta^{15}N$ values by Liz Dixon, Rothamsted Research-North Wyke. All samples were dried and ground to a fine powder before being accurately weighed into tin capsules (enough sample to contain 50-70 µg N) then crimped. Standards used for analysis calibration depended on whether the samples were natural abundance or enriched with ^{15}N (values are shown in the Table 2.3). Samples were analysed by an elemental analyser which consisted of a Carlo Erba CN NA2000 analyser (Milan, Italy) linked to a SerCon 20-22 isotope ratio mass spectrometer (SerCon Ltd, Crewe, UK).

Table 2.3. Standards used for analysis and for calibration of results

Standard	% N	% C	^{15}N (δ_{air})	^{13}C (δ_{VPDB})
IA R001- flour	1.88	40.20	2.55	-26.43
AQC 206 - flour	1.78	41.57	2.76	-25.80
AQC 38 - soil	0.22	5.54	251.9	-25.68
AQC 209 - grass	1.95	42.76	6795	-28.60

2.4. Extraction, isolation and derivatisation of hydrolysable amino acids- Compound specific stable isotope analysis

Methods presented in this section for compound specific stable isotope analysis of AAs have been developed in the Organic Geochemistry Unit at the University of Bristol, therefore, these methods are already established and published elsewhere, for example; Corr et al. (2007) and Styring et al. (2012).

2.4.1. Reagents

All solvents used were of HPLC grade and purchased from Rathburn Chemicals Ltd, except for acetyl chloride, triethylamine and acetic anhydride which were purchased from Sigma-Aldrich (Dorest, UK).

2.4.2. Internal and external amino acid standards

An internal standard (IS) (200 and 400 µg mL^{-1}) for hydrolysable amino acid (AA) analysis was prepared using norleucine (Nle) 0.1 M hydrochloric acid (HCl, reagent grade). The external standard (1 mg mL^{-1}) consisted of a mixed solution of 14 AAs [Alanine (Ala), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), hydroxyproline (Hyp), leucine (Leu), lysine (Lys), Norleucine (Nle), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr) and valine (Val)] (Sigma-Aldrich, Poole, UK) in 0.1 M hydrochloric acid (HCl, reagent grade). The external standard of AAs was used to monitor instrument function, aid in the identification of AAs by retention time, calculate the AA response factors for quantification (Section 2.5.1), and also providing quality control (QC) to regulate the performance of the gas chromatograph-combustion-isotope ratio mass spectrometer (GC-C-IRMS, Section 2.4.8.3). Due to this later use of the external standard, the ^{15}N isotopic composition of all these AAs (apart from Hyp and Nle) had been previously determined by elemental analysis-isotope mass spectrometry (EA-IRMS) by Thermo-Fisher Scientific in Bremen, Germany and the Merlewood/Lancaster node of the NERC Life Sciences Mass Spectrometry Facility (LSMSF) using the primary reference material NIST 8547 IAEA-N-1 ammonium sulfate ($(NH_4)_2SO_4$), $\delta^{15}N$ +0.4 ‰. For the use of the external standard in this application, the mixed standard (0.5 mL) was derivatised in culture tubes alongside samples following the methods described in Section 2.4.7.

2.4.3. Lipid extraction of plant material

Prior to AA extraction, plant material only needed to be lipid extracted. Plant material was weighed into culture tubes (see Table 2.4) with a 2:1 *v/v* solution of DCM: methanol (5 mL) and was sonicated for 15 mins. Samples were then centrifuged at 3000 rpm for 10 minutes and the supernatant removed, before repeating the process a further time. The residue solvent was then removed from the sample, at 40°C, under a gentle stream of N_2, before being AA extracted.

2.4.4. Amino acid extraction

Freeze-dried material (Table 2.4, plant material underwent lipid extraction prior to this step Section 2.3.3) was placed into culture tubes and the weight recorded. An internal standard of Nle was added depending on the sample being extracted (Table 2.4). Hydrolysis through addition of 6 M HCl (5 mL) to each tube, was carried out for 24 h at 100°C, where tubes were sealed under oxygen free N_2 with a lid and polytetrafluorethylene (PTFE) tape, this minimised oxidative degradation of AAs under these harsh conditions. During this time samples were vortex mixed four times. Samples were then allowed to cool before being centrifuged at 3000 rpm for 10 minutes, the supernatant was then transferred to 28 mL vials. The residue was then washed with 0.1M HCl (2 mL) and vortex mixed before being centrifuged (3000 rpm, 10 minutes). The supernatant was combined in the 28 mL vial before being dried at 60°C under a gentle stream of N_2 and stored in a freezer at -20°C in 0.1 HCl (1 mL).

Table 2.4. Corresponding material weights and volume of internal standard used

Material	Weight (mg)	Amount of internal standard (norleucine)
Plant exudates	All available freeze-dried sample	50 µl of 200 µg mL^{-1}
Plant material	10-15	150 µl of 400 µg mL^{-1}
Soil	100	100 µl of 400 µg mL^{-1}

The acid hydrolysis conditions described results in the complete conversion of glutamine to glutamic acid (Glx) (or glutamate) and asparagine to aspartic acid (Asx) (or aspartate) (Fountoulakis and Lahm, 1998), so where glutamic acid and aspartic acid are referred to within this thesis, a combined mixture with the amide derived analogues is implied.

2.4.5. Preparation of Dowex resin

Dowex resin (50WX8 200-400 mesh ion exchange resin, Sigma-Aldrich, Dorset, UK) was prepared so that all cation exchange sites were occupied by H^+ ions, this was achieved by soaking in 3M NaOH overnight (12 h), the excess NaOH was removed and the resin was washed and agitated in DDW five times (shaking the bottle, then decanting the DDW once settled). The resin was then soaked and stored in 6M HCl for at least 24 hours prior to use.

2.4.6. Purification of hydrolysable amino acids by cation exchange column chromatography

Cation exchange chromatography was used to isolate AAs from the hydrolysates using the prepared Dowex resin. Dowex resin (~1 mL) was pipetted into a flash column and washed with DDW (3 x 2 mL) using N_2 gas to flush the column until neutral pH was obtained and confirmed with universal indicator paper. At all stages in the washing process the column was not allowed to completely dry out. The hydrolysed sample was then applied to the top of the column (1 mL in 0.1 M HCl) and washed with double distilled water (2 x 2 mL). The AAs were then collected in clean culture tubes by elution with 2M ammonium hydroxide (NH_4OH) (4 x 2 mL). Purified AAs were then dried at 60°C under a gentle stream of N_2 then frozen.

2.4.7. Amino acid derivatisation

Due to the polar nature of AAs, they require derivatisation to *N*-acetyl-*O*-isopropyl esters (NAIP) to increase their volatility. Derivatisation of AAs was carried out according to the methods by Corr et al. (2007). Isopropylation of AAs is achieved through addition of 0.25 mL of a mixture (4:1 *v/v*) of isopropanol: acetyl chloride (AC; puriss. p.a. grade) to each purified AA fraction in a culture tube. The isopropanol: acetyl chloride mixture was prepared in an ice bath (dropwise addition of acetyl chloride to ice-cold isopropanol), tubes were then sealed with PTFE tape and heated at 100°C for 1 hour. Each tube was then placed in the freezer at −20°C for at least 5 minutes to quench the reaction. After which excess solvent was evaporated under a gentle flow of N_2 at 40°C. Samples were re-dissolved in DCM (2 × 0.25 mL) and excess solvent removed along with any residual water under a gentle steam of N_2.

Acetylation was then carried out using a fresh mixture of acetone: triethylamine: acetic anhydride (triethylamine ≥99.50 % purity, acetic anhydrideReagentPlus® grade) (5:2:1 *v/v/v*), 1 mL of which was added to each sample. Each tube was capped then sealed with PTFE tape and heated at 60°C for 10 minutes. Excess reagents were then evaporated under a very gentle stream of N_2 at room temperature. AAs were then re-dissolved in ethyl acetate (2 mL) and saturated NaCl (1 mL) and the mixture vortexed to allow phase separation. After settling the organic layer was removed into a 7 mL vial. The NaCl was then further washed with ethyl acetate and the above procedure repeated 3 times to ensure all AAs derivatives were removed. The combined organic phases were then evaporated gently under N_2 at room temperature.

Residual water was removed by adding DCM (3 x 1 mL) and evaporating the solution gently under N_2 in an ice bath. Samples were capped then sealed with PTFE tape and stored in the freezer at −20°C until analysis.

2.4.8. Instrumental analyses

2.4.8.1. Gas chromatography- Flame Ionisation Detection (GC-FID)

A Hewlett-Packard Series II 5890 GC (Agilent Technologies, Santa Clara, CA, USA) was used to perform all analyses. The carrier gas was He and the gas chromatograph was operated under constant pressure. Each sample (1 µL) was injected onto a DB-35 (35%-phenyl)-methylpolysiloxane, mid-polarity coated capillary column (60 m x 0.32 mm i.d., 0.5 µm phase thickness; Agilent Technologies). The temperature programme used was: initial temperature was held at 70 °C for 2 min, then increased to 150°C at 15°C min^{-1}, then to 210°C at 2°C min^{-1} and finally to 270 °C at 8°C min^{-1} and held for 5 min.

The flame ionisation detector (FID) was used for the quantification of individual AAs (Section 2.5.1) by comparison with the IS Nle. AAs were primarily identified by their known elution order and comparison to the external standard of AAs which was derivatised for assigning peaks by comparison of retention times. All data acquired was collected and analysed using Clarity software (version 2.6.2.226, DataApex Ltd, Prague, Czech Republic).

Part way through the project the GC was updated to a 7890B Agilent Technologies GC system, with data collected in Chapters 5 and 6 using this new instrument, as well as the majority of the data in Chapter 4 (excluding at the investigation of transfer between plants in rhizotrons, Section 4.3.1). While the column and the temperature programme remained the same, the GC operated under constant flow. The GC was also upgraded with an autosampler, reducing the need for manual injections, apart from when sample volumes were insufficient. Data was collected and analysed online and offline, as appropriate, through Chemstation (version C.01.07, Agilent Technologies, Santa Clara, USA).

2.4.8.2. Gas chromatography- Mass Spectrometry (GC-MS)

The retention times and identification of AAs were confirmed by GC-MS, with the external standard and a selection of samples being checked. To do this, the GC-MS was set-up similarly to the GC-FID and GC-C-IRMS, with the same temperature programmes and column type used (DB-35, coated capillary column 60 m × 0.32 mm i.d., 0.5 µm phase thickness; Agilent Technologies) with He carrier gas. A Thermo-Scientific Trace 1300 gas chromatograph interfaced to a Thermo-Scientific ISQ single quadrupole mass spectrometer *via* a heated transfer line was used. Samples were automatically injected using a Thermo-Scientific AS 1310 autosampler *via* a split-splitless inlet set to splitless mode. The mass spectrometer was operated in electron ionisation (EI) mode. Data was acquired and analysed using Xcalibur (version 3.0, Thermo-Fisher Scientific). AA NAIP esters were identified by molecular ion and by the loss of characteristic fragments (laboratory manual).

2.4.8.3. Gas Chromatography- Combustion- Isotope Mass Spectrometry (GC-C-IRMS)

A ThermoFinnigan Delta^{Plus} XP IRMS (Thermo-Scientific, Bremen, Germany) was used to determine the $\delta^{15}N$ values of derivatised AAs. The MS (operating in EI mode, 100 eV, three Faraday cup collectors for *m/z* 28, 29 and 30) was interfaced to a Trace 2000 GC *via* a Combustion III interface. The oxidation reactor consisted of high purity copper (Cu >99.9%) and nickel (Ni > 99.6%) wires (OEA Laboratories, Cornwall, UK) and was held at 1030 °C. Samples (1 µL) were injected using an auto-sampler in the majority of cases (CTC Analytics GC Pal auto-sampler), with low volume samples requiring manual injection, injected samples were introduced using a programmable temperature vaporisation (PTV) injector held at 200°C. The carrier gas used was He at a flow rate of 1.4 mL min^{-1} and the MS source pressure was maintained at 9×10^{-4} Pa. AA separation was accomplished using a DB-35 capillary column (30 m × 0.32 mm i.d., 0.5 µm film thickness; Agilent Technologies). GC oven temperature started at 40°C and was held for 5 min before heating at 15°C min^{-1} to 120°C, at 3°C min^{-1} to 180°C, at 1.5°C min^{-1} to 210°C and finally at 5°C min^{-1} to 270°C and held for 1 min. A cryogenic liquid nitrogen trap was employed to remove CO_2 from the oxidised and reduced analytes.

All the $\delta^{15}N$ values are reported relative to a reference N_2 gas of known N isotopic composition, previously calibrated against the AIR international isotope standard, introduced directly into

the ion source *via* an open split in four pulses at the beginning and end of each run (Figure 2.7). The AA external standard mixture (Section 2.3.2) of known $\delta^{15}N$ values was run before and after each sample (in duplicate sample runs) to monitor the performance of the instrument and was used to report the analytical error (1σ), representing the standard deviation of the $\delta^{15}N$ values in each standard. The $\delta^{15}N$ values of the standard were accepted and hence the sample run when at least 75% of the AA $\delta^{15}N$ values in the standard were within ±1‰ and the rest within ±1.5‰. All data collected were analysed using Isodat (version 3.0, Thermo-Scientific). As no N atoms were added as a result of the derivatisation process no correction of determined $\delta^{15}N$ values was needed.

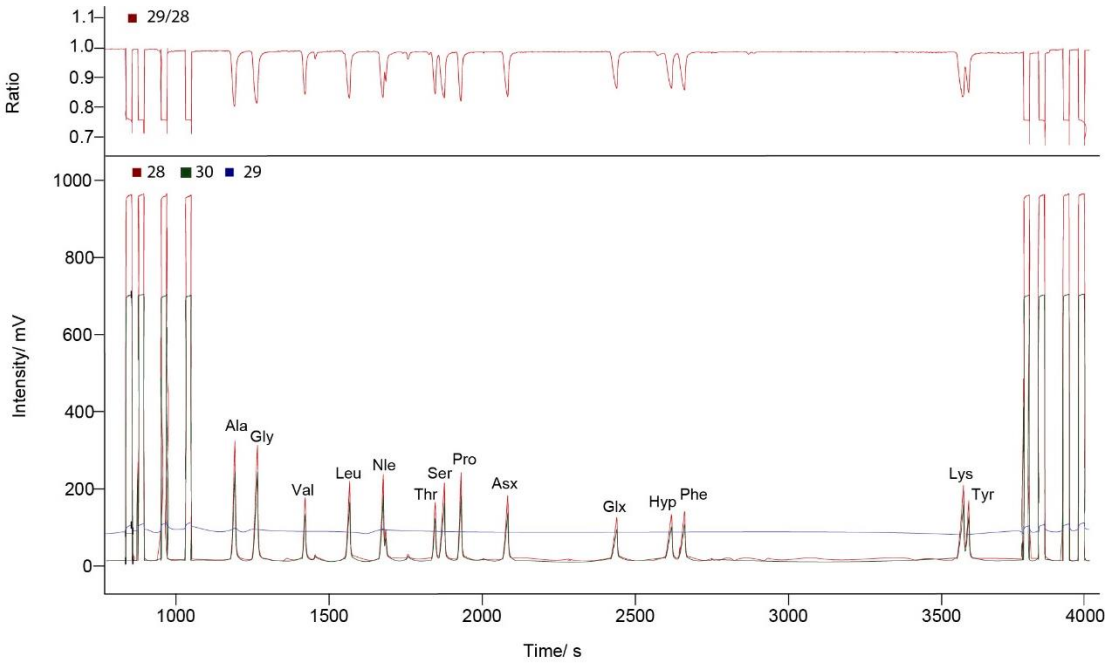


Figure 2.7. Typical GC-C-IRMS chromatogram of an *N*-acetyl-*O*-isopropyl derivatised AA standard showing the ion current signals recorded by the GC-C-IRMS operating for N_2 (m/z 28, 29 and 30) (bottom panel) and the ratio of m/z 28 to 29 which is used to generate $^{15}N/^{14}N$ isotope ratios (top panel).

2.5. Data processing, calculations and statistical tests

2.5.1. Quantification of amino acids

AA quantification was determined by GC-FID using the IS Nle. Due to structural differences between AAs, their FID response factors vary, meaning that equivalent concentrations of the IS and AAs will not provide the same GC peak area. Therefore, FID response factors (RFs) are determined for each AA relative to the IS in order to calculate the AA concentration in a sample (Table 2.5).

$$\text{Sample AA apparent mass} = \left(\frac{\text{Sample AA peak area}}{\text{Sample IS peak area}}\right) \times \text{ mass of IS}$$

Equation 2.1

$$\text{FID RF} = \frac{\text{Standard AA peak area}}{\text{Stanard I. S. peak area}}$$

Equation 2.2

Table 2.5. FID response factors (RF) for quantifying AAs by GC-FID. Average of all standards run throughout the project.

Amino acid	FID response factor (RF)	Amino acid	FID response factor (RF)
Ala	0.91	Phe	1.14
Asx	0.91	Pro	0.87
Glx	0.85	Ser	0.89
Gly	0.86	Thr	0.55
Hyp	0.83	Tyr	1.03
Leu	1.03	Val	0.63
Lys	0.73		

$$\textit{S}\text{ample AA actual mass} = \text{Sample AA appatent mass} \times \text{ FID RF}$$

Equation 2.3

The actual mass of the sample weighed at the beginning of the AA extraction (Section 2.4.4) can then be used to give the concentration of the AAs in the sample in mg of AA per gram of sample (mg g^{-1}).

2.5.2. Equations relating to ^{15}N isotopic composition

There are two ways that the isotope ratio ($^{15}N/^{14}N$) can be expressed in studies, in terms of absolute or relative units; both have been used in ^{15}N enrichment studies. Relative units, i.e. $\delta^{15}N$ values, are used to measure small differences in ^{15}N natural abundance or low relative amounts of ^{15}N enrichment requiring a high degree of precision. As $\delta^{15}N$ values do not vary linearly with isotopic enrichment, absolute units (^{15}N abundance, atom %) are used when samples are artificially enriched with ^{15}N, and the difference between natural abundance samples and ^{15}N enriched samples is substantial (Chalk et al., 2014).

The absolute unit gives the value in the absolute number of atoms of a given isotope in 100 atoms of the total element, and is based on the atom or isotope-amount fraction of ^{15}N relative to total N.

$$^{15}\text{N Abundance (atom \%)} = \left(\frac{\text{number of } ^{15}\text{N atoms}}{\text{number of } ^{14}\text{N} + ^{15}\text{N atoms}}\right) \times 100$$

Equation 2.4

Often in ^{15}N enrichment studies, this is expressed as atom % excess (APE), this gives the level of isotopic abundance above the background reading. As the natural abundance of ^{15}N in the atmosphere is constant at 0.3663 ± 0.0004 % (Mariotti, 1983) this is often used as the background level, such as in the work of Janzen and Bruinsma (1989). However, more suitable backgrounds are those of unlabelled materials used within the particular study (i.e. control samples). Schmidtke (2005) expressed the importance of choosing the most appropriate background ^{15}N, either from a non-nodulated reference plants or a legume grown on soil without ^{15}N application, with a range of different backgrounds being chosen with studies, however, precise descriptions are often missing (Jensen.,1996a; Khan et al., 2002a; Mayer et al., 2003). Here the background ^{15}N has been chosen as the corresponding unlabelled sample, i.e. if calculating the atom % ^{15}N excess of clover root, an unlabelled clover root was used as the background.

$$^{15}\text{N enrichment}\left(\text{atom \% } ^{15}\text{N excess}\right) = {}^{15}\text{N}_{\text{sample}} - {}^{15}\text{N}_{\text{background}}$$

Equation 2.5

The relative unit involves the measurement of the isotope ratio of the (Number of ^{15}N) atoms)/(Number of ^{14}N atoms) of a sample and of the standard of atmospheric N_2. Therefore, this measurement is the relative difference in isotope ratios compared to the internationally agreed measurement standard, AIR-N_2 (Mariotti, 1983), which acts as a scale anchor. Where by definition 0‰ ~ 0.0036765 $^{15}N/^{14}N$ ~ 0.336 atom % ^{15}N.

$$\delta^{15}N\ (‰) = \left(\frac{^{15}N/^{14}N_{\ sample} - {}^{15}N/^{14}N_{\ standard}}{^{15}N/^{14}N_{\ standard}}\right) X\ 1000$$

Equation 2.6

Equation 2.6. can also be expressed slightly differently, using the R-values of the isotope ratios ($^{15}N/^{14}N$).

$$\delta^{15}N\ (‰) = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) \times\ 1000$$

Equation 2.7

The two units are not strictly convertible due to the different scales (Chalk, 1995), although it is possible using the technique employed in this research. Where $R_{standard}$ is the $^{15}N/^{14}N$ ratio of AIR-N_2, which is the isotopic standard for N (i.e. 0.0036765) and the relative units are in $\delta^{15}N$ values (‰).

$$R_{sample} = R_{standard} \times \left[\left(\frac{\text{Relative units}}{1000}\right) + 1\right]$$

Equation 2.8

$$\textit{A}\text{bsoulte units (atom \%)} = \left(\frac{R_{sample}}{R_{sample} + 1}\right) X\ 100$$

Equation 2.9

2.5.3. Bulk nitrogen isotopic compositions and percentage incorporation of applied ^{15}N-label

The percentage incorporation of the applied ^{15}N-label into bulk δ^{15}N values of soil or plant material is estimated by firstly calculating the atom fraction (AF) (as described by Knowles et al., 2010).

$$AF = \frac{R_{standard} \times \left(\frac{\delta^{15}N}{1000} + 1\right)}{1 + \left(\frac{\delta^{15}N}{1000} + 1\right)}$$

Equation 2.10

Simply, this can be calculated by firstly converting the bulk δ^{15}N value (relative units) to a ratio (R_{sample}) as in Equation 2.8, then to an atom fraction (AF), before calculating the atom fraction excess (AFE) where the background is the AF of control samples.

$$AF = \left(\frac{R_{sample}}{R_{sample} + 1}\right)$$

Equation 2.11

$$AFE = AF_{sample} - AF_{background}$$

Equation 2.12

The total amount of ^{15}N present in moles per gram of bulk sample can then be calculated as per Knowles (2009), where %TN is the total nitrogen content of the sample in percentage terms. This also represents the number of moles of ^{15}N in the sample (soil or plant material) at a given time, otherwise known as applied ^{15}N retained (N_R).

$$N_R = AFE \times \left(\frac{\%TN}{1400}\right)$$

Equation 2.13

The number of moles of N applied into the system can then be calculated (N_A), where $RFM_{nitrogen}$ is the N percentage content of the substrate applied (i.e. if $CO(^{15}NH_2)_2$ 46.8%), S is the mass of the substrate applied per gram of sample and $Mass_{nitrogen}$ is the average mass of nitrogen applied, i.e. if at 10 atom % then $Mass_{nitrogen}$ is 14.1.

$$N_A = \frac{RFM_{Nitrogen} \times S}{Mass_{Nitrogen}}$$

Equation 2.14

The percentage incorporation of the applied ^{15}N-label into bulk δ^{15}N values of soil or plant material can then be calculated, where AFE_A is the AFE of applied N (assuming natural abundance is 0.0036765, i.e. if at 10 atom %, AFE_A is 0.1-0.003765), therefore this calculates the value of the number of moles of excess ^{15}N applied above the natural abundance values for the substrate.

$$\%\ incorporation\ into\ bulk\ \delta^{15}N = \left(\frac{N_R}{N_A \times AFE_A}\right) \times 100$$

Equation 2.15

2.5.4. Percentage incorporation of applied ^{15}N-label into amino acids

The percentage incorporation of the applied ^{15}N-label into AAs takes into account both the concentration of each AA and the ^{15}N enrichment of the AAs. Therefore, this calculation shows how much of the applied ^{15}N-label was incorporated into individual AAs at x concentration and is ^{15}N enriched by y ‰ (Charteris et al., 2016). Similarly, to above (Equations 2.8, 2.11, 2.12) the AFE for each AA needed to be calculated first. The number of moles of N in each AA can then be calculated (n_N) for each AA, where $Conc_{AA}$ is the AA concentration in g, $Mass_{nitrogen}$ is the average mass of nitrogen applied, and RFM_{AA} is the relative formula mass of each AA. Where the AA molecular structure only contains one N atom (this is the case for 13 out of 14 AAs studied), n_N is the equal to the number of moles of the AA in the sample, but double if the AA contains two N atoms (e.g. in lysine).

$$n_N = \frac{Conc_{AA} \times \frac{N\ atom \times\ Mass_{Nitrogen}}{RFM_{AA}}}{Mass_{Nitrogen}}$$

Equation 2.16

From the AFE and n_N, the ^{15}N enrichment of the individual AAs (E) can then be calculated. Further to this, the sum of E (if considered to represent the whole soil protein pool) can represent the newly synthesised plant or soil protein at that particular time (Charteris et al., 2016).

$$E = n_N \times AFE$$

Equation 2.17

This may then be expressed in terms of the percentage of the applied ^{15}N into each AA as in Knowles et al. (2010). Where N_A is the number of moles of ^{15}N applied as calculated in Equation 2.14.

$$\%\ incoroporation = \left(\frac{E}{N_A}\right) \times 100$$

Equation 2.18

2.5.5. Percentage retained ^{15}N-label in bulk material incorporated into amino acids

The percentage incorporation (Equation 2.18) is affected by the amount of ^{15}N-label within the system, as over-time ^{15}N is incorporated into the plant-soil system, taken up by plants or lost out of the system and may skew results as less ^{15}N is available to be incorporated. Furthermore, in the experiments conducted within this project, the applied ^{15}N-label was not always applied directly to the material being studied, for example, when looking at soil AAs the ^{15}N was applied through the plant system. Therefore, the percentage incorporation at time t based on the moles of applied ^{15}N retained (above the control values) which is based on bulk ^{15}N values can be calculated in order to help make comparisons between different experimental periods, this is known as the percentage retained. The latter calculation also enables understanding of how much of the ^{15}N present in the bulk soil or plant is within the AAs. However, it could also

be argued that the reduction in ^{15}N available due to loss or incorporation of ^{15}N into the plant-soil system is just another process competing against AA biosynthesis, therefore, this should not be discounted and hence, within this thesis, comparisons are made between the two calculations.

The percentage retained uses the bulk $\delta^{15}N$ values of the particular material being studied (plant or soil) to firstly calculate the applied ^{15}N retained (N_R) as in Equation 2.13, which represents the number of moles of ^{15}N in the sample (soil plant material) at a given time.

$$\% \text{ retained} = \left(\frac{E}{N_R}\right) \times 100$$

Equation 2.19

2.5.6. Calculations relating to N-transfer between plants

2.5.6.1. Yield-dependent expressions

N-transfer can be expressed in a number of ways, either as the amount, a proportion or as a percentage of legume N or non-legume N. A number of calculations can be performed to describe N-transfer, therefore it is important to be precise about which terms are being used. Ledgard et al. (1985) estimated the proportion of legume N transferred to the non-legume by directly labelling the legume with ^{15}N followed by analysis of the plant material harvested. This method estimates the ratio between the ^{15}N-label in the non-legume (grass) and the total ^{15}N-labelled N in both the legume (clover) and non-legume (grass). Where $P_{transfer}$ is the proportion of legume N transferred to the non-legume, to calculate this ^{15}N $content_{non\text{-}legume}$ and ^{15}N $content_{legume}$ can be firstly calculated through Equation 2.21.

$$P_{transfer} = \frac{^{15}\text{N Content}_{non-legume}}{^{15}\text{N Content}_{non-legume} + ^{15}\text{N Content}_{legume}}$$

Equation 2.20

To calculate the ^{15}N content, the atom % ^{15}N excess in the plant (legume or non-legume) or soil is calculated through the atom % ^{15}N in the enriched sample minus that of background atom % ^{15}N in the control of the corresponding sample (as previously calculated in Equation 2.5). For the atom % ^{15}N excess for the legume and non-legume weighted mean ^{15}N enrichment of the shoots and roots at final sampling (or harvest) have been used to calculate the total atom% ^{15}N excess for the whole plant. The atom % ^{15}N excess for legume can also be substituted for the ^{15}N enrichment in the roots at harvest (atom % ^{15}N $excess_{legumeR}$) in order to represent the actual amount of N available for transfer. Total $N_{plant\ or\ soil}$ denotes the N-yield of the sample, for plant (legume or non-legume) this is expressed in mg plant^{-1}.

$$^{15}\text{N Content}_{\text{plant or soil}} = \text{atom \%}^{15}\text{N excess}_{\text{plant or soil}} \text{ X Total N}_{\text{plant or soil}}$$

Equation 2.21

From Equation 2.20, the amount of legume N transferred to the non-legume ($N_{transfer}$) can then be calculated.

$$\text{N}_{\text{transfer}} = \text{P}_{\text{transfer}} \times \text{ Total N}_{\text{legume}}$$

Equation 2.22

The proportion of N in the non-legume derived from the transfer of legume N (Ndft) can then be calculated, which can either be expressed as a proportion as shown in Equation 2.23, or converted to a percentage.

$$\text{Ndft} = \frac{\text{N}_{\text{transfer}}}{\text{Total N}_{\text{non-legume}}}$$

Equation 2.23

If the legume root (legumeR) is used as a better approximation of the amount of N available for transfer and used to calculate Equation 2.20 and 2.21, similarly Equation 2.22 can be substituted for the Total $N_{legumeR}$ aiding Equation 2.24 to give $Ndft_R$. This method assumes that

the N deposited in the rhizosphere by the legume and taken up by the receiving grass plant, had the same ^{15}N enrichment during the labelling periods as the legume root at the time of harvest.

$$\mathrm{Ndft_R} = \frac{\mathrm{N_{transfer}}}{\mathrm{Total\ N_{non-legume}}}$$

Equation 2.24

However, Equation 2.20 assumes that there are no losses of ^{15}N from the soil-plant systems, and that all the ^{15}N-label absorbed by the labelled plant part results in labelling of all N compounds which can be transferred. Furthermore, this assumes there is no significant transfer to the soil N pool with Equation 2.20 and will overestimate the proportion of legume N transferred to the non-legume, therefore, the non-simplified version is shown in Equation 2.25 (Ledgard et al., 1985), which can then be used to calculate Ndft as in Equation 2.23.

$$\mathrm{P_{transfer}} = \frac{^{15}\mathrm{N\ Content_{non-legume}}}{^{15}\mathrm{N\ Content_{non-legume}} + ^{15}\mathrm{N\ Content_{legume}} + ^{15}\mathrm{N\ Content_{soil}}}$$

Equation 2.25

Similarly, Equation 2.25 can be modified, and legume root (legumeR) can be substituted into the equation and followed through in Equation 2.22 to give $Ndft_r$ in Equation 2.24.

$$\mathrm{P_{transfer}} = \frac{^{15}\mathrm{N\ Content_{non-legume}}}{^{15}\mathrm{N\ Content_{non-legume}} + ^{15}\mathrm{N\ Content_{legumeR}} + ^{15}\mathrm{N\ Content_{soil}}}$$

Equation 2.26

2.5.6.2. Yield-independent expressions

Similarly, for a uniformly labelled legume the proportion of N in the non-legume derived from the transfer of legume N (Ndft) can be calculated through yield-independent expressions using solely the measurement of atom % ^{15}N excess (as previously calculated in Equation 2.5).

$$\mathrm{Ndft} = \frac{\text{atom \%}^{15}\text{N excess}_{\mathrm{non-legume}}}{\text{atom \%}^{15}\text{N excess}_{\mathrm{legume}}}$$

Equation 2.27

However, labelling methods frequently result in non-uniform distribution of the label, resulting in an over-estimation of the proportion of the legume N transferred to the non-legume. Giller et al. (1991) proposed that the ^{15}N enrichment of the legume roots (atom % ^{15}N excess$_{\mathrm{legumeR}}$) at harvest may better represent the ^{15}N enrichment of the N transferred (as shown in the yield independent expressions), and similarly Ndft$_r$ can be calculated.

$$\mathrm{Ndft_r} = \frac{\text{atom \%}^{15}\text{N excess}_{\mathrm{non-legume}}}{\text{atom \%}^{15}\text{N excess}_{\mathrm{legumeR}}}$$

Equation 2.28

2.5.7. Percentage N-transfer from plants to soil

The percentage of N transferred from labelled plant roots to the soil may be calculated through the expression described by Jansen and Bruinsma (1989), which is ultimately used to express N derived from rhizodeposition (Ndfr), the atom % ^{15}N excess can be calculated as in Equation 2.5.

$$\mathrm{Ndfr} = \frac{\text{atom \%}^{15}\text{N excess}_{\mathrm{soil}}}{\text{atom \%}^{15}\text{N excess}_{\mathrm{root}}} \times 100$$

Equation 2.29

2.5.8. Statistical analysis

All data were subjected to one or two-way analysis of variance (ANOVA) for testing significant differences between parameters, this was performed using IBM SPSS Statistics (version 24, International Business Machines Corporation, Portsmouth, UK). Differences were considered to be significant when $P \leq 0.05$, and Tukey post-hoc tests were used to establish where the differences lie.

All data were visually inspected before performing statistical tests and descriptive statistics performed with SPSS. Outliers were identified using the function with SPSS and with the use of Dixon's Q test, using a critical Q-value at a confidence level of 95%, where the null hypothesis can be rejected if the calculated experiment Q-value is greater than the critical Q-value. Where an outlier, was identified the whole sample set was excluded.

Chapter 3

Developing a method for the application of a ^{15}N-label into white clover (*Trifolium repens*) to study nitrogen transfer

3. Developing a method for the application of a ^{15}N-label into white clover (*Trifolium repens*) to study nitrogen transfer

3.1. Introduction

^{15}N-labelling techniques are commonly used to study N cycling as well as for estimating N-transfer from legume to non-legume in intercropping systems. More recently ^{15}N tracers have been employed in studies to assess belowground N and to quantify rhizodeposition in terms of N, where the fate of N from ^{15}N-labelled plants into the soil is determined. Typically, "rhizodeposition is the process of release of organic and inorganic compounds from living plant roots" (Wichern et al., 2008). These compounds can be volatile, non-particular (passive or diffused root exudates, secretions) and particular (root border cells, slough epidermal roots, root hairs, root fragments) (Wichern et al., 2008), which is important in determining the different forms in which N can be transferred between plants.

There are a variety of methods which have already been established to ^{15}N-label legume plants, these include exposure to atmospheric $^{15}N_2$ or $^{15}NH_3$, shoot application of ^{15}N or labelling through the root system (Table 3.1), with the shoot-labelling technique being the most commonly used (Chalk et al., 2014). All methods available have their drawbacks, with the introduction of ^{15}N either not following the natural physiological pathway of assimilation or the use of artificial conditions (Wichern et al., 2008). Ideally, isotopic labelling would result in uniform labelling of the whole plant (Fustec et al., 2010).

There are several methods which can be used to introduce the ^{15}N-label into the shoots, i.e. through leaf feeding by immersion of the leaf in the labelling solution, spraying over the leaves, petiole or stem feeding through a wick or even injecting directly into the stem (Table 3.1). The idea of applying the ^{15}N-label to part of the plant shoots is that the ^{15}N-label is transferred to all organs by the sap stream (Fustec et al., 2010). Leaf-labelling techniques can only be used after the first leaf has emerged, allowing only pulse or multiple pulse-labelling (Wichern et al., 2008). Ledgard et al. (1985) first developed the method for using foliar ^{15}N absorption and compared different methods of applying the ^{15}N substrate to the shoots to directly calculate N-transfer in laboratory and field experiments, this enabled transfer to be calculated over a relatively short time-scale.

The root system has a limited number methods for introducing a ^{15}N-label, it can be achieved either through a split-root technique (where the roots are divided into two visibly equal parts), applying the ^{15}N-label to the adventitious roots or transplanting plants after initial growth in a ^{15}N medium (Table 3.1). Applying the ^{15}N-label to the root system allows the natural N pathway of uptake to be followed (Wichern et al., 2008). The use of the split-root technique was first noted by van Kessel et al. (1985) to study N-transfer between two plants, but has also been used in single plant systems to investigate N loss from root systems (Sawatsky and Soper, 1991). All of these methods (shoot or root ^{15}N enrichment) can be used to determine N-transfer between plants, working on the assumption that detection of ^{15}N enrichment in the plant tissues of the accompanying non-legume plant is evidence of N-transfer (Jensen, 1996b; Chalk and Smith, 1997).

A number of studies have been conducted that compared the different ^{15}N enriching techniques (Jensen, 1996b; Merbach et al., 2000; Chalk et al., 2002; Khan et al., 2002a, b; Hertenberger and Wanek, 2004; Yasmin et al., 2006; Mahieu et al., 2007; Wichern et al., 2008; Fustec et al., 2010; Chalk et al., 2014). These studies showed that the "best method" of ^{15}N application not only depended on the species being studied but the aims of the project. For example, whether it was to study N-transfer, rhizodeposition, quantify belowground N or determine the long-term fate of plant derived N in soil (Merbach et al., 2000). Khan et al. (2002a) studied four different legume species, showing that ^{15}N-labelling *via* the leaf-flap was best for fababean (*Vicia faba*), mungbean (*Vigna radiate*) and pigeonpea (*Cajanus cajan*), but petiole feeding was best for chickpea (*Cicer arietinum*). This shows that even though methods are established they still need to be evaluated to identify which is the most appropriate for a given experiment, as it is known that one technique is not necessarily applicable to all legumes (Khan et al., 2002a).

Table 3.1. Examples of previous methods for estimating the transfer of fixed N from legumes to companion species using ^{15}N *

Method	Substrate	Plant Species	Reference
Atmospheric labelling			
Two-litre incubation chamber with control atmosphere for 72 hours	$^{15}N_2$	Common bean (*Phaseolus vulgaris)*	Ruschel et al., 1976
Short exposure of plants to atmosphere (6 h intervals during the growing season)	$^{15}NH_3$ at 22.193 atom %, (released through the addition of dissolved $(^{15}NH_4)_2SO_4$ into NaOH	Wheat (*Triticum aestivum* var Leader)	Janzen and Bruinsma, 1989
Incubation in enriched atmosphere to 19 to 21 days	$^{15}N_2$ enriched atmosphere (using 99.7 atom % to enrich the atmosphere resulting in 3 to 6 atom %)	White clover (*Trifolium repens* L. cv. Blanca) and perennial ryegrass (*Lolium perenne* L. cv. Trani) Alder (*Alnus glutinosa* L.) saplings with red fescue (*Festuca rubra* L.)	McNeill et al., 1994
Plants harvested 24 days after first exposure to ^{15}N enriched atmosphere	$^{15}NH_3$ generated by the addition of a $(^{15}NH_4)_2SO_4$ solution (95 atom %) to NaOH, final concentration of 200 µL L^{-1} NH_3	Wheat (*Triticum aestivum* L. cv Mario, CV. Star)	Merbach et al., 2000
Enclosing of the root system only of clover in atmosphere for 3 days and measuring uptake in accompanying plant	$^{15}N_2$ atmosphere with 300 ml $^{15}N_2$ injected (99 atom %)	White clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.)	Lesuffleur et al., 2013

Shoot labelling			
1. Three trifoliate leaves were immersed in substrate[1] for 48 h; 2. Three petioles (trifoliolate leaves removed) were immersed in substrate[1] for 48 h; 3. Three trifoliolate leaves were immersed in second subsrate[2] for 48 h	$K^{15}NO_3$ 30 mM at 95 atom % for first three methods[1], $(^{15}NH_4)_2SO4$ 15 mM at 99 atom %[2]	Subterranean clover *(Trifolium subterraneum* L. *Woogenellup*) associated with ryegrass (*Lolium rigidum* Gaud.)	Ledgard et al., 1985
1. Spreading substrate on to the surface of the unifoliolate leaves; 2. Immersing the leaves into bottles of substrate 3. Injecting substrate into the petioles	$(^{15}NH_4)_2SO_4$ 30 mM at 99 atom %	Common bean (*Phaseolus*) associated with maize (*Zea mays* L.)	Giller et al., 1991
Immersion of the leaves and petioles in substrate for 72 h	$K^{15}NO_3$ 30 mM at 99.7 atom %	White clover (*Trifolium repens* L. cv. Menna)	Murray and Hatch, 1994
Cotton-wick inserted into a hole in the stem and linked at the other end to a reservoir of labelled	^{15}N urea (500 µL of a solution containing 2 mg excess urea ^{15}N was fed to each plant)	Lupin (*Lupinus angustifolius* cv. Danja)	Russell and Fillery, 1996a, b
Laterally injected into the stele between the cotyledonary and unifoliate node	$^{15}NH_4CI$ (95 atom %) (20 µl ^{15}N solution with 0.8 mg ^{15}N excess per plant)	Cowpea (*Vigna unguiculata* L. Walp)	Götz and Herzog, 2000
1. Leaf-flap feeding of ^{15}N 2. Petiole feeding of ^{15}N 3. Multiple ^{15}N feeding events	^{15}N urea concentrations were used (0.1, 0.5, 1, 2% w/w) at 0.2 mL $plant^{-1}$	Fababean (*Vicia faba*), chickpea (*Cicer arietinum*), mungbean *(Vigna radiata*) and pigeonpea (*Cajanus cajan)*	Khan et al., 2002

Root labelling (Split root)			
Split-root with plants harvest 48 h after first ^{15}N application	$(^{15}NH_4)_2SO_4$ 0.7 mM at 99.99 atom %	Soybean (Glycine max [L.] Merr) associated with maize (*Zea mays* L.)	Van Kessel et al., 1985
Split-root with plants being harvest 28 d after first ^{15}N application	$K^{15}NO_3$ (2.44 mg of ^{15}N at 50 atom %)	Berseem (*Trifolium alexandrinum* L. cv. Landsorte) associated (*Zea mays* L. cv. Honeycomb-Fl)	Frey and Schüepp, 1993
Split-root with ^{15}N application applied weekly for four weeks with harvest one week after final application (~ 35 d)	$K^{15}NO_3$ weekly over four weeks (total of 40 mg at 99.7 atom %)	Pea (Pueraria phaseoloides) and rubber tree (Hevea brasiliensis)	Ikram et al., 1994
Split-root with plants being harvested after 28, 35 and 45 d after initial labelling depending on experiment	$K^{15}NO_3$ 5 mM at 10 atom %	Peas (*Pisum sativum* L. cv. Bodil) and spring barley (*Hordeum vulgare* L. cv. Nery)	Jensen, 1996
Root labelling (transplanting)			
Legumes plantlets raised on a coarse sand and watered with ^{15}N Hoagland's solution for 22 days, after transplantion harvested after 7, 12 or 15 weeks	$K^{15}NO_3$ 12.5 mM (atom % not detailed)	Soybean (*Glycine max* L. cv. Maple Arrow) (Legume) associated with maize (*Zea mays* L. Cv. Pioneer 3809)	Hamel et al., 1991
Both plants grown with a ^{15}N nutrient solution (replaced every 15 days) plants were transplanted after 104 days of growth, after transplantation plants were harvest at 50 and 79 days	$K^{15}NO_3$ (54.8 atom %, 0.1001g N L^{-1})	Brome grass (*Bromus riparius*) and alfalfa (*Medicago sativa* L.)	Tomm et al., 1994
Grown on sand culture irrigated with ^{15}N nutrient solution before being transplanted (variable time), plants harvested between 15 and 312 days	$^{15}NH_4{}^{15}NO_3$ at 10 atom % with varying application rate (between 2.5 to 7.3 g N m^{-2})	Pea (*Pisum sativum* L. cv. Capella), oat (*Avena sativa*), red clover (*Trifolium pratense*) and timothy (*Phleum pretense*)	Carlsson and Huss-Danell, 2014

* Not exhausted list of sampling studies, just examples of different plant species, methods and labelling source

Each of the methods reported have been developed for a particular purpose, with each having its own advantages and limitations (Khan et al., 2002a; Wichern et al., 2008). The method selected often depends on the morphology of the plant species to be studied, the growing conditions available, i.e. greenhouse, poly-tunnel or field (Mahieu et al., 2007) as well as the aims of the study in question. Palta et al. (1991) noted that an important first step in studying any plant-nitrogen relationship is to identify the methodologies which can be used to appropriately enrich the plant with ^{15}N, therefore, this chapter aims to address establishing a suitable method.

For the purpose of this work, a select number of methods were chosen to be studied in detail. The use of atmospheric labelling either from the use of $^{15}N_2$ or $^{15}NH_3$ to the whole plant or just exposing the root nodules has not been discussed due to its short-term use (hours to days), the associated problems of maintaining plants in a sealed air-tight environment as well as the high costs involved. Due to this, the technique is not widely used (Chalk et al., 2014). However, atmospheric labelling is the only method for directly determining the amount of N_2-fixed which is transferred between plants (Frey and Schüepp, 1992). Furthermore, not all shoot labelling techniques are applicable to clover, for example, the cotton-wick stick application cannot be used on thin-stemmed plant species, only woody species like lupin (*Lupinus* spp.) (Russell and Fillery, 1996; Yasmin et al., 2006). Spraying ^{15}N over the leaves has not been reported as a very effective method, leading to high losses of the ^{15}N-label and runoff into the soil (Russell and Fillery, 1996). Two more viable comparable techniques were chosen to be studied in detail: leaf-labelling through submersion and root-labelling through the split-root technique.

3.2. Objectives

The overall aim of this chapter was to establish the best method for applying ^{15}N compounds to clover plants for the ensuing work. Each experiment presented in this chapter builds on the findings and outcomes of the previous experiment in order to arrive at a viable method. The work presented in this chapter provides the foundation for further chapters, producing a robust method for tracing N-transfer through plant-soil systems. This chapter will provide more details and insights into different methods available for ^{15}N enriching plants.

The specific objectives of this work were to:

(i) Compare the uptake of ^{15}N into different plant parts over time using different ^{15}N labelling techniques in rhizotrons.

(ii) Monitor the response of the total hydrolysable root AA concentrations to different ^{15}N additions to clover plants in rhizotrons and determine whether the application method effects the root AA ^{15}N distribution.

(iii) Monitor the total hydrolysable AA concentrations and ^{15}N AA distributions in different plant parts and soils over time after shoot ^{15}N application in rhizotrons.

(iv) Determine the effect soil background N has on ^{15}N enriching plant parts.

(v) Determine the most appropriate labelling substrate solution to use for ^{15}N enriching plant parts.

(vi) Determine and quantify the exudation of AAs from clover and whether the labelling solution used effects exudation.

(vii) Quantify the recovery rate of AAs from sand.

(viii) Determine the allocation of ^{15}N to individual AA exudates.

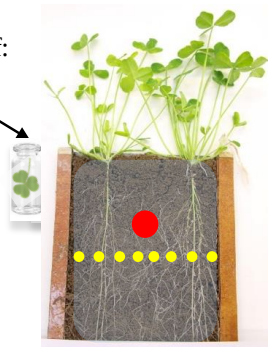
(ix) Determine the suitability of the use of a split-root labelling technique in incubation tubes compared to leaf-labelling in rhizotrons.

This chapter describes results from four individual experiments on clover plants, with outcomes from each experiment informing the subsequent experiment (Figure 3.1). Further to this, an AA recovery study from sand was conducted to establish the recovery rate from sand when spiked with a mixed AA standard, ultimately to inform experiments where AA exudates were quantified.

Comparison of different ^{15}N labelling techniques in rhizotrons

1 mM $^{15}NH_4{}^{15}NO_3$ at 10 atom %

Comparison of:
Control
Spot
Multi
Spray
Leaf



Aims:
- Determine the best methods for applying N compounds
- Determine the time needed for N uptake into clover foliage
- Determine if application methods affects the AA ^{15}N distribution in the roots

Verification of the leaf-labelling technique in rhizotrons

30 mM $^{15}NH_4{}^{15}NO_3$ at 10 atom %

Aims:
- Verify previous findings on the leaf labelling technique
- Determine the time needed for N uptake via the leaf labelling technique by sampling whole rhizotrons over time
- Determine the effect of time on the AA ^{15}N distribution

^{15}N uptake through leaf-labelling technique by eliminating background N

30 mM $^{15}NH_4{}^{15}NO_3$ at 98 atom %
or $CO(^{15}NH_2)_2$

Aims:
- Determine the effect of eliminating soil N uptake
- Determine if the labelling substrate ($^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$) effects the level of enrichment in the plant
- Determine which AAs are exudated from clover
- Determine if the labelling substrate effects the exudation of AAs from clover

Suitability of split-root labelling technique

30 mM $^{15}NH_4{}^{15}NO_3$ at 98 atom %
or $CO(^{15}NH_2)_2$

Aims:
- Determine if root uptake of ^{15}N results in higher plant ^{15}N enrichment
- Determine if the labelling substrate ($^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$) effects the level of enrichment in the plant
- Determine which AAs are exudated from clover
- Determine if the labelling substrate effects the exudation of AAs from clover

Figure 3.1. Summary and aims of experiments conducted in this chapter.

3.3. Materials and methods

3.3.1. Comparing ^{15}N-labelling techniques in rhizotrons

Five seeds of white clover (*Trifolium repens*) cv. Aber Dai were placed across the top of each of the prepared rhizotrons (Section 2.2.9) (for this experiment the mother clover plant had yet to be established). The seeds were covered with soil to a depth of 0.5 cm, rhizotrons were then watered to give an approximate soil water content of 60%. A plastic bag was placed over the surface of the rhizotrons to reduce water loss, which were then placed at room temperature to germinate. After two weeks, any surplus germinated clover plants were removed to give exactly two plants per rhizotron. Rhizotrons were kept in growth cabinets with a controlled environment at 20/15ºC day/night with a 16 h photoperiod. Clover plants were allowed to grow for a further four weeks before all plants were trimmed to an approximate height of 3 cm, this helped to reduce growth allowing time for the experiments to be set up. The plant biomass collected from trimming the plants was freeze-dried and bulk δ^{15}N values determined.

After a further two weeks of growth, plants were labelled with 1 mM $^{15}NH_4{}^{15}NO_3$ at 10 atom %. The substrate (1 mL) was applied to each rhizotron through either a single point injection into the centre of the rhizotrons ("spot"), multiple injections using an 8-channel pipette ("multi"), sprayed over the surface of the soil ("spray") or applied by submerging leaves into the solution ("leaf") (Figure 3.2). For the rhizotrons where the leaves were submerged in the solution, one attached leaf from each clover plant was immersed in ^{15}N enriched solution in 7 mL glass vials, which were taped onto the front of the rhizotrons. Care was taken to avoid any contact with the rest of the plant, after 72 hours the immersed leaves were cut and removed with the vials (as previous carried out by Ledgard et al., 1985 and Murray and Hatch, 1994). For each of these different application systems four replicate rhizotrons were used, including a control where no solution was applied. The base of each rhizotron was placed in a plastic bag to avoid mixing of the soil solution.

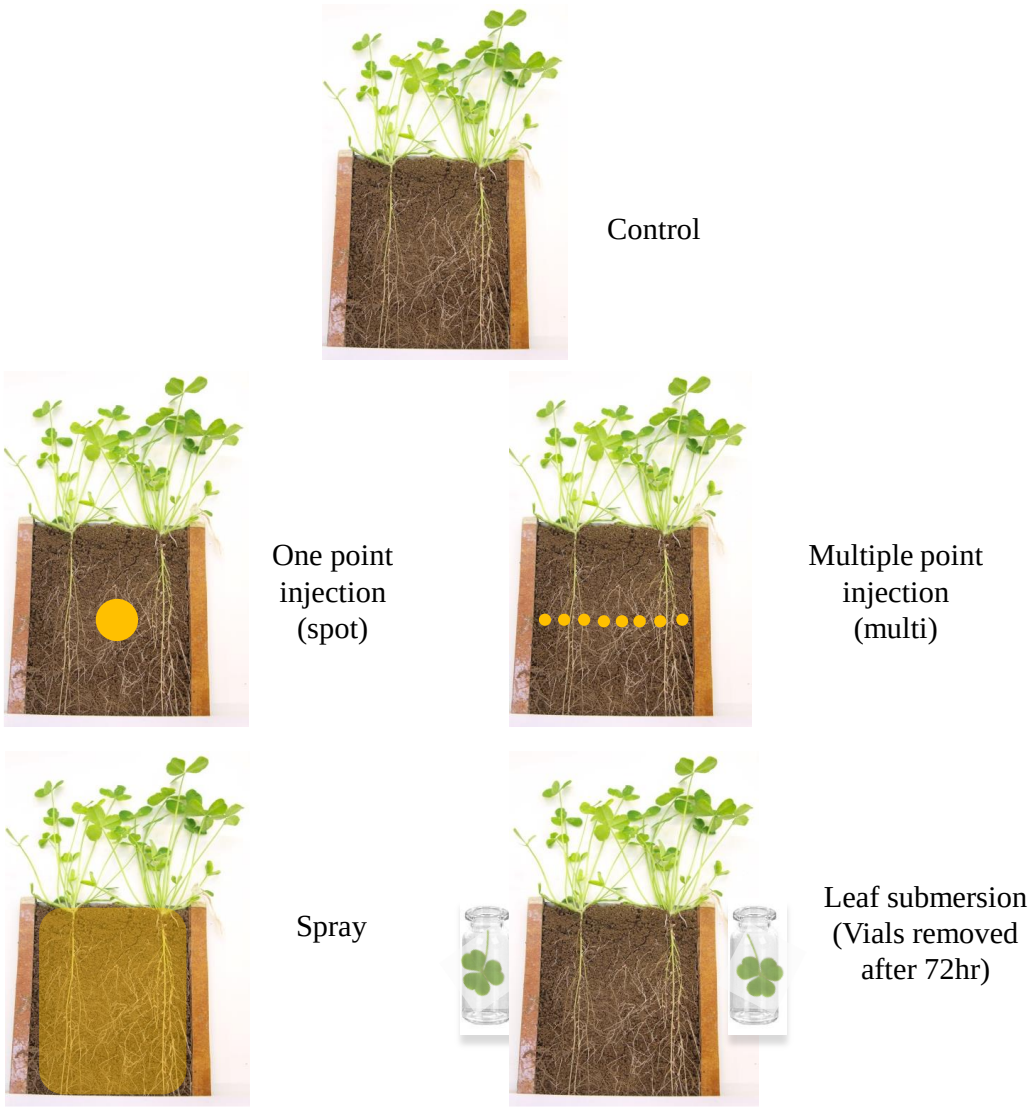


Figure 3.2. Experimental set up to compare different labelling techniques

Clover plants were then sampled 1 h after the labelling period, where one fully unfolded leaf was removed from the end of a growing point in each rhizotron. Subsequent sampling was undertaken at 2, 3, 6, 24, 30, 54, 100, 174 and 361 h. After sampling all plant material was immediately placed in a drying oven at 80ºC for 24 h before being weighed. Individual leaf samples were crushed to a fine powder before being weighed out for analysis.

After the experimental time period (361 h), each rhizotron was deconstructed and plant parts separated into leaves, stolon, flowers and roots before being placed in a drying oven at 80ºC for 24 h and then weighed. Large plant parts were ground into a fine powder using a ball mill grinder, while small plant parts (flowers) were ground using plastic vials. Three soil samples were collected from the top, middle and bottom of each rhizotron to establish if there were any

differences in where the labelled solution had been applied. Soils were then placed in the drying oven at 30ºC for one week. Soils were ground using a pestle and mortar and then passed through a 150 micron sieve to ensure a uniform fine powder. Bulk $\delta^{13}C$ and $\delta^{15}N$ values were determined for each sample (Section 2.3). In addition, AAs were also extracted (root samples only) from the deconstructed rhizotrons. Extracted AAs were subsequently derivatised and analysed by GC-FID (quantification) and GC-C-IRMS (compound specific $\delta^{15}N$ value determination) (Section 2.4).

3.3.2. Verifying the leaf-labelling technique in rhizotrons

Rhizotrons were set up as described in Section 2.2.9, before two clover cuttings from the end of a stolon growing point were taken from the mother plant described in Section 2.2.5 and planted in the top of each rhizotron. Rhizotrons were watered with a modified Hewitt solution (Section 2.2.7) twice a week and allowed to grow for 4.5 weeks in the glasshouse before the experiment commenced. The experiment was conducted between November and December 2014, where average temperatures were 19.8-20.0°C, maximum and minimum temperatures can be found in Section 2.2.5. Before ^{15}N-labelling commenced four random rhizotrons were sampled to give background nitrogen levels (time 0). Each clover plant was labelled using the leaf labelling technique as described in Section 2.2.9, however, only one attached clover leaf from each plant was submerged in the labelled solution (30 mM $^{15}NH_4{}^{15}NO_3$ at 10 atom %). Rhizotrons were then sampled and deconstructed at 1 h after the labelling period, then subsequently 2, 3, 6, 24, 30, 54, 100, 174 and 361 h with four repeats per time point. At each sampling point all plant parts were recovered and divided into leaves, roots and stolon; a soil sample was also collected from each rhizotron. Samples were immediately frozen by being submerged within a glass vial into liquid nitrogen before storing in the freezer at −20°C. All samples were then freeze-dried and ground into a fine powder using a pestle and mortar. Samples were analysed for bulk $\delta^{13}C$ and $\delta^{15}N$ value determination (Section 2.3). In addition, AAs were also extracted from a selection of time points (0, 24, 100 and 361 h) for leaves, stolon, roots and soil, derivatised and analysed by GC-FID (quantification) and GC-C-IRMS (compound specific $\delta^{15}N$ value determination) (Section 2.4).

3.3.3. Eliminating background nitrogen and ^{15}N uptake through leaf-labelling

Rhizotrons were set up similarly to Section 2.2.9, however, 200 g of acid washed furnaced sand (Section 2.2.4) was used to fill rhizotrons instead of soil. One clover cutting from the end of a stolon growing point was taken from the mother plant described in Section 2.2.5 and planted in the top of each rhizotron. The experiment was conducted between January and April 2015, where average temperatures were 19.6-21.6°C, maximum and minimum temperatures can be found in Section 2.2.5. Rhizotrons were watered daily with dilute nutrient solution for the initial growing period (6 weeks) (Section 2.2.7) and then, latterly, with a full-strength nutrient solution due to reduced growth in the sand. After a total of seven weeks of growth, six replicate rhizotrons were labelled through the leaf labelling technique (as described in Section 2.2.9) with 30 mM $^{15}NH_4{}^{15}NO_3$ at 98 atom % or 30 mM $CO(^{15}NH_2)_2$ at 98 atom % along with DDW as a control. 100 h after labelling had commenced, each rhizotron was deconstructed, sand collected from and plant parts separated into leaves, stolon and roots. All samples were placed immediately in a drying oven at 80ºC for 24 h and then weighed. All samples were analysed for bulk $\delta^{13}C$ and $\delta^{15}N$ value determination (Section 2.3).

3.3.4. Amino acid recovery from sand

Acid washed furnaced sand (Section 2.2.4) (80 g) was spiked with a mixed AA standard of known concentration (100 µL of 1 mg mL^{-1}, Section 2.4.2), with a blank sand acting as a control, receiving only internal standard spike (Nle, 100 µL of 1 mg mL^{-1}). Samples were then suspended on filter paper and flushed through using 80 mL of DDW, before being frozen and later freeze-dried. To compare the recovery, 80 mL of DDW only was spiked with the mixed AA standard and underwent the freezing and freeze-drying process. Subsequently, AAs were extracted from the sand, derivatised and analysed by GC-FID (quantification) (Section 2.4). The mixed AA standard, without being freeze-dried, was also subjected to the same process for comparative purposes. Each treatment comprised three replicates.

3.3.5. Determining the suitability of the split-root labelling technique and quantifying plant exudations

Incubation tubes were setup as described in Section 2.2.10, with one tube of sand acting as the labelling compartment (LC) and only one further tube of soil for the transfer compartment (TC). Cuttings of clover plants only were taken and allowed to grow in a pot of compost for three weeks to allow the roots to establish before inserting into the glass Y-tubes (growing period between July to August 2016, average temperature can be found in Section 2.2.5). Clover roots were divided between the labelling and transfer compartments. Plants in incubation tubes were moved into the contaminant section of the greenhouse, to reduce the likelihood of whitefly infestations during the running of the experiment (which were a problem in the greenhouse at the time). Plants were allowed to grow for a further three weeks before the experiment commenced. During this time, the average temperature during the running of the experiment was 22.8°C, with a minimum of 17.2°C and maximum of 34.5°C.

After three weeks of growth, the substrates were introduced into the LC by injecting with DDW for the control or 30 mM $^{15}NH_4{}^{15}NO_3$ or 30 mM $CO(^{15}NH_2)_2$ at 98 atom % (0.2 mL x 5) with five repeats per treatment (details about injecting the substrate can be found in Section 2.2.10). Incubation tubes were sampled at the 100 h labelling period. After the experimental time period, plant leaves were cut immediately to halt any further transfer. For the labelling compartment, AAs were leached from the sand (with roots still intact) using 40 mL DDW. Following this, each incubation tube was deconstructed and plant parts and soils separated. All samples were immediately placed in the freezer then later freeze-dried and weighed. All plant and soil samples were analysed for bulk $\delta^{13}C$ and $\delta^{15}N$ value determination (Section 2.3). In addition, AAs were also extracted from the soils and exudates, derivatised and analysed by GC-FID (quantification) and GC-C-IRMS (compound specific $\delta^{15}N$ value determination) (Section 2.4).

3.4. Results

3.4.1. Comparison of different ^{15}N-labelling techniques in rhizotrons

Results in this section address the objective (i) set out in section 3.2.

3.4.1.1. Leaf uptake of ^{15}N

Each different application method for applying ^{15}N showed a different enrichment pattern over-time (Figure 3.3), with a significant difference being found between treatments and time intervals ($F_{36,149}$=2.065, P=0.001). However, all treatments showed an initial drop in δ^{15}N values when applying ^{15}N enriched compounds. Following this, the spot and multi treatments were seen to produce increasing δ^{15}N values throughout the experiment, showing an increasing uptake of the ^{15}N-label from the soil and allocation to the leaves. Due to the similarity in application of the spot and multi treatments, no significant difference was found between these two treatments. Figure 3.3 shows little variation between δ^{15}N values for the control and spray treatments, with no significant difference being found. This showed that the spray treatment was an ineffective method for applying the ^{15}N-label to the plant, probably due to too much dilution of the ^{15}N-label over the soil surface, resulting in the low enrichment values seen. Furthermore, the leaf treatment was shown to significantly differ from all other treatments (Table 3.2). Interestingly, δ^{15}N values obtained for the leaf treatment only showed a trend of increasing after 30 h and peaked at 100 h before values decreased again. For this treatment one leaf from each plant was submerged in the ^{15}N-label for 72 h, results showed that this method takes longer for enrichment in the plant parts to commence due to this being a slower uptake route compared to when the ^{15}N-label was applied directly to the soil as in the spot and multi treatments. The lag in uptake of the leaf treatment can be seen from the fact that the δ^{15}N values take a further few hours to decrease after the source of label is removed at 72 h. Also, for this treatment it is not known how much δ^{15}N values would have increased if the source was not removed. Similarly, the spot and multi treatments showed an increasing trend in δ^{15}N values and it is not known how much more samples would have become enriched if the experiment had continued.

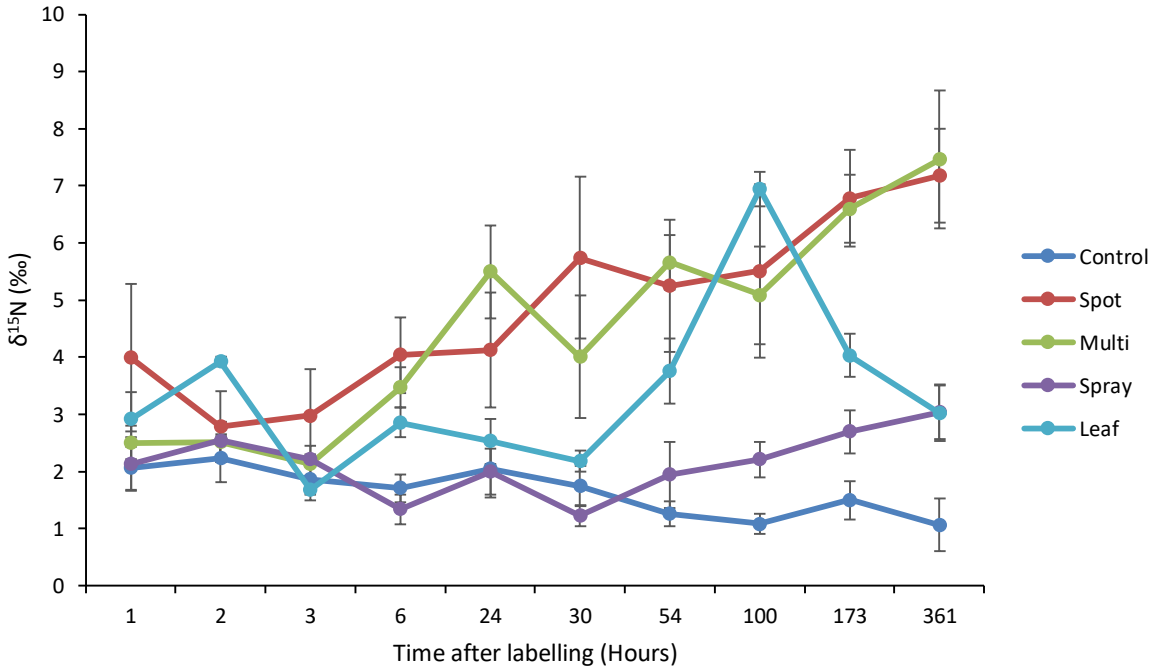


Figure 3.3. Temporal changes after labelling with ^{15}N (1 mM NH_4NO_3 at 10 atom %) in $\delta^{15}N$ values of the first unfolded leaf at the end of a growing point of white clover (*Trifolium repens*). (mean ± standard error; n=4)

Table 3.2. Statistical results for experiment looking at the temporal changes in $\delta^{15}N$ values of the first unfolded leaf at the end of a growing point of white clover (*Trifolium repens*).

Statistical test	Interaction	P-value
Two-way ANOVA	Time * Treatment	P=0.001
	Time	P=0.000
	Treatment	P=0.000
Post hoc	Spot * Multi	NS
	Control * Spray	NS
	Leaf * Control/ Spot/ Multi/ Spray	P< 0.021

NS: main effect or interaction not significant at the P<0.05 level.

3.4.1.2. Partitioning of ^{15}N into plant parts

Deconstruction of each rhizotron at the end of the experimental period showed a significant difference between treatment and different plant parts ($F_{8,45}$ = 15.594, P= 0.000) (Figure 3.4) (Table 3.4). Results showed elevated $\delta^{15}N$ values in the roots of the spot and multi treatments, this is unsurprising due to the ^{15}N-label being applied onto these plant parts, however, this also showed the non-uniform distribution of label within these treatments. This finding is reinforced by no significant difference being found between $\delta^{15}N$ values in the stolon, leaves, and flowers but the $\delta^{15}N$ values of these plant parts all significantly differed from the roots (P = 0.000). However, Figure 3.4 showed that $\delta^{15}N$ values for the control were generally higher in the roots than for the other plant parts. Similarly, as with the temporal changes in $\delta^{15}N$ values in individual leaves (Figure 3.3), no significant difference was found between the spot and multi treatments. Additionally, Figure 3.4 compared to Figure 3.3 showed more elevation of $\delta^{15}N$ values of the spray treatment compared to the control, especially in the roots where the treatment was applied. The results for the $\delta^{15}N$ values of the final biomass produced by the spray treatment in Figure 3.4 also differed from results in Figure 3.3, as Figure 3.4 showed no difference in the $\delta^{15}N$ values of the spray and leaf treatments. Crucially, results showed the most uniform distribution of the ^{15}N-label throughout different plant parts for the leaf treatment. Furthermore, the $\delta^{15}N$ values of different plant parts in the leaf treatment were likely to have been higher if the rhizotron was deconstructed and sampled at the peak of $\delta^{15}N$ values in the leaves (100 h, Figure 3.3).

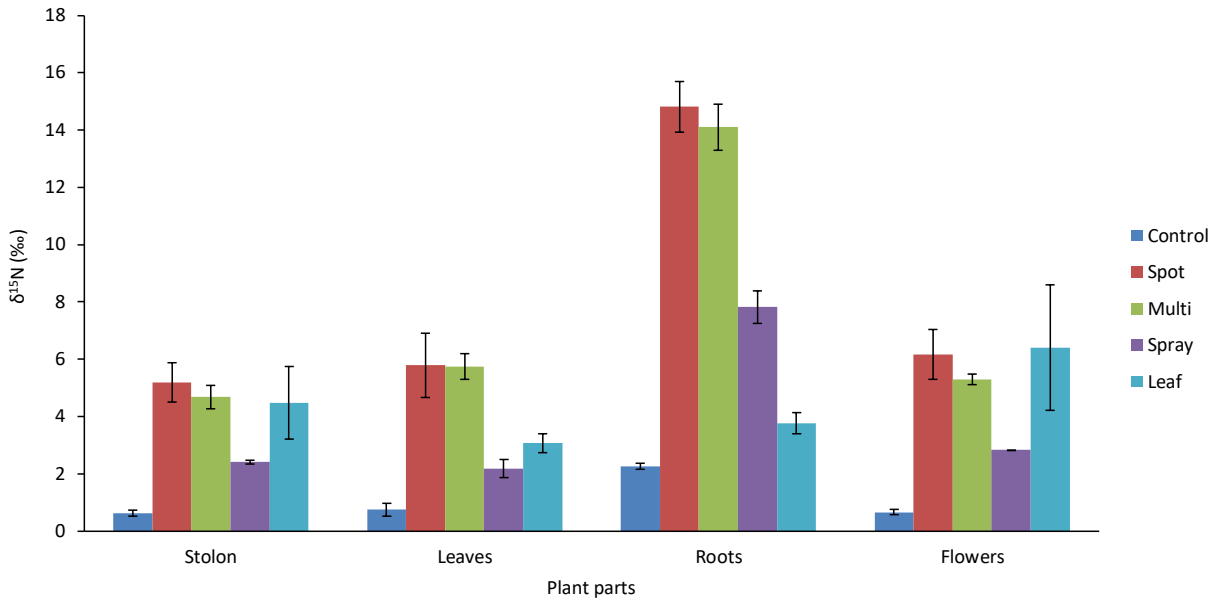


Figure 3.4. $\delta^{15}N$ values of different plant parts of white clover (*Trifolium repens*) in each treatment at the end of the experiment period (361 h), (mean ± standard error; n= 4, however not all plants produced flowers).

Table 3.3. Statistical results for experiment looking at the temporal changes in $\delta^{15}N$ values of the first unfolded leaf at the end of a growing point of white clover (*Trifolium repens*).

Statistical test	Interaction	P-value
Two-way ANOVA	Plant part * Treatment	P= 0.000
	Plant part	P=0.000
	Treatment	P=0.000
Post hoc	Control * Spot/ Multi/ Spray/ Leaf	P=0.000
	Spot * Multi	NS
	Spray * Leaf	NS

NS: main effect or interaction not significant at the P<0.05 level.

The percentage incorporation of the applied ^{15}N-label into different plant parts, was found to be surprisingly high despite the relatively low enrichment of $\delta^{15}N$ values (Table 3.4). For the stolon, results varied between 4.46-9.62%, for the leaves between 3.68-13.06% for all treatments. However, no difference was found between the percentage incorporation into the stolon or leaves for different treatments. Higher incorporation was attained in the roots, especially for the treatments where the ^{15}N-label was directly applied, with a maximum incorporation of 27.80% being achieved. A significant difference in percentage incorporation was found between different treatments ($F_{3,12}$= 61.900, P=0.000), where the spot and multi

treatments were found to be similar to each other but different from the spray and leaf, which also differed from each other. With a high percentage incorporation of the applied ^{15}N-label being found into bulk δ^{15}N values, this implies that in order to achieve overall higher δ^{15}N values then either the concentration of the applied ^{15}N-label needed increasing or the atom %.

Table 3.4. Percentage incorporation of applied ^{15}N-label (1mM NH_4NO_3 at 10 atom %) via different application methods into bulk plant δ^{15}N values (%) of white clover (*Trifolium repens*), (mean ± SEM). One-way AVONA result comparing the effect of different application methods on the percentage incorporation of the ^{15}N-label into different plant parts.

	Stolon	Leaves	Root
Spot	27.2 ± 3.1	45.5 ± 9.7	93.4 ± 4.8
Multi	23.7 ± 4.4	45.2 ± 8.5	80.1 ± 5.9
Spray	15.2 ± 1.8	12.7 ± 3.0	41.7 ± 5.0
Leaf	25.6 ± 7.9	21.7 ± 4.6	11.3 ± 2.7
ANOVA	NS	NS	P=0.000

NS: main effect or interaction not significant at the P<0.05 level.

Due to the long growing period in the comparison of ^{15}N-labelling methods experiment, each rhizotron produced a substantial amount of biomass, averaging a total of 2300-2500 mg compared to subsequent experiments in this chapter [Table 3.5, compared to verification of the leaf-labelling technique (3.9) and eliminating background N (3.13) experiments]. The different ^{15}N application methods compared were not found to have an effect on plant growth and therefore final biomass (Table 3.5). No significant difference was found between the total biomass produced in each rhizotron, or the N content for each plant part in different treatments. This finding is fundamental as it showed that δ^{15}N values produced and findings of this initial investigation are not due to differences in plant growth resulting from the treatment.

Table 3.5. Dry matter and N-content for white clover (*Trifolium repens*) plant parts sampled after a 361 h labelling study comparing different ^{15}N-labelling methods: control, spot, multi, spray or leaf with $^{15}NH_4{}^{15}NO_3$ (1 mM at 10 atom %) (mean ± standard error; n=4). One-way ANOVA result comparing the effect of different application methods on the resultant plant dry matter and N content.

	Dry matter (mg plant^{-1})				N content (mg plant^{-1})		
	Stolon	Leaves*	Roots	Total**	Stolon	Leaves	Roots
Control	654 ± 45.3	705 ± 58.3	735 ± 20.8	2392 ± 65.5	16.8 ± 1.7	18.4 ± 1.4	17.5 ± 0.6
Spot	726 ± 34.4	595 ± 53.0	694 ± 52.0	2349 ± 30.2	12.7 ± 1.6	18.9 ± 0.8	16.2 ± 0.9
Multi	755 ± 29.4	713 ± 42.2	740 ± 50.3	2531 ± 67.9	14.6 ± 1.0	19.1 ± 0.5	16.4 ± 1.0
Spray	770 ± 69.2	735 ± 38.1	834 ± 19.7	2581 ± 53.7	18.4 ± 1.5	19.7 ± 1.8	19.6 ± 0.4
Leaf	670 ± 60.4	725 ± 10.7	720 ± 69.5	2516 ± 18.3	15.6 ± 2.0	17.2 ± 1.2	17.0 ± 1.3
ANOVA	NS	NS	NS	NS	NS	NS	NS

*Leaf dry matter only includes leaves sampled at the end of the experimental time period and not those removed during the time course experiment

**Total includes all leaves removed at sampling time intervals and any flowers produced

NS: main effect or interaction not significant at the P<0.05 level.

3.4.1.3. Uptake of ^{15}N into bulk soil

Soil samples collected at the end of the experiment did not show much enrichment, even when ^{15}N was directly applied, with a maximum enrichment of 9.21‰ being achieved. However, a significant difference was found in $\delta^{15}N$ values for both treatment and location of soil sampling (Figure 3.5) ($F_{8,45}$= 12.521, P =0.000). Results showed elevated $\delta^{15}N$ values for the middle soil section of the spot and multi treatments, this was expected due to where the ^{15}N-label was applied. The middle soil section $\delta^{15}N$ values were significantly different from the top or bottom. The results showed that the $\delta^{15}N$ values for the baseline, control and leaf did not significantly differ from each other, but each of these treatments had $\delta^{15}N$ values significantly different from the spot, multi and spray treatments.

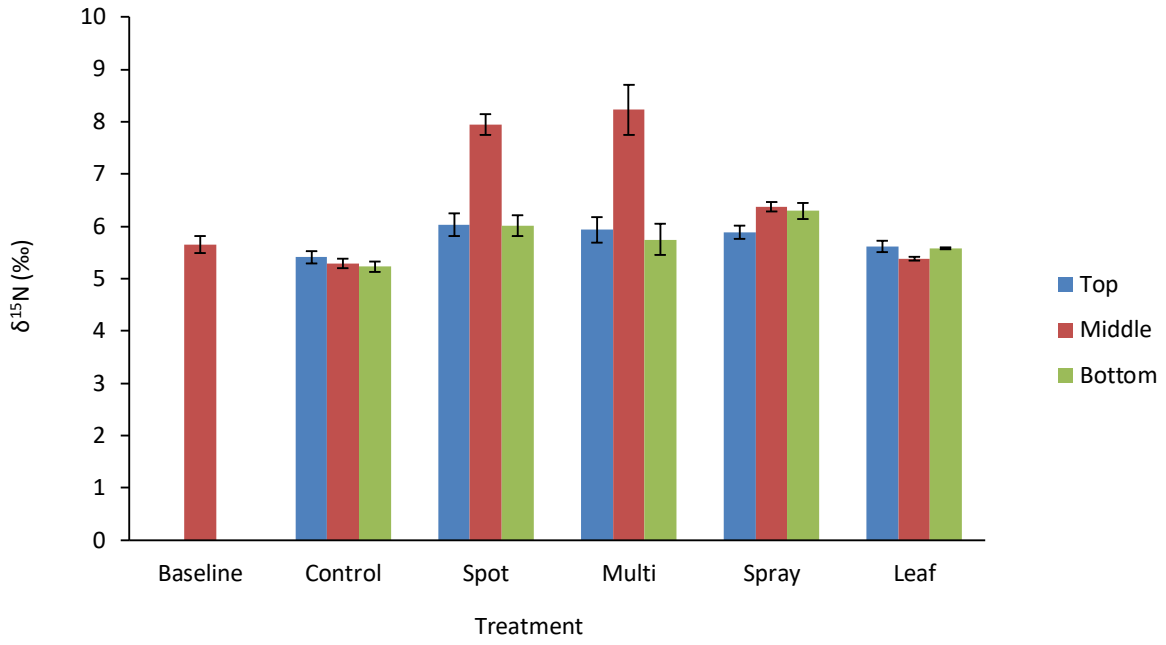


Figure 3.5. $\delta^{15}N$ values of soils in each treatment, taken from the top, middle and bottom of each rhizotron at the end of the experimental period with white clover (*Trifolium repens*) (mean ± standard error; n=4). Baseline represents a sample of soil which was not used in the experiment and has had no plants growing in it.

Similarly to the bulk $\delta^{15}N$ values of different plant parts, high incorporation of the applied ^{15}N-label was observed for the bulk soil $\delta^{15}N$ values (Table 3.6). However, this showed that increasing the $\delta^{15}N$ value of the soil, even when the label was applied directly to the soil, was more difficult than increasing the $\delta^{15}N$ value of plant parts. The percentage incorporation

values of the ^{15}N-label into the bulk soil (middle section) of different treatments were seen to significantly differ from each other ($F_{3,12}$= 25.244, P=0.000), where the spot and multi treatments were different from the spray and leaf (which were similar to each other). The total N and C contents of the soils were also found to be consistent throughout the experiment with different treatments (Table 3.6). The N content varied between 0.463 and 0.512%, and for C between 4.747 and 4.649% for the middle section soil, with similar values being found for the top and middle sections of the rhizotron soil.

Table 3.6. Incorporation of applied ^{15}N-label (1mM $^{15}NH_4{}^{15}NO_3$ at 10 atom %) via different application methods into bulk soil δ^{15}N values (%), mean soil total N (% TN) and total C (%TC) in the middle of the rhizotron only (mean ± SEM). One-way AVONA result comparing the effect of different application methods on the percentage incorporation of the ^{15}N-label into the soil, %TN and %TC.

	Incorporation of ^{15}N-label into bulk soil (%)	% TN	% TC
Control	NA	0.473	4.47
Spot	180 ± 13.6	0.499	4.18
Multi	196 ± 32.9	0.491	4.30
Spray	70.5 ± 5.99	0.480	4.51
Leaf	5.72 ± 2.72	0.478	4.48
ANOVA	P=0.000	NS	NS

NS: main effect or interaction not significant at the P<0.05 level.

3.4.2. Effect of different ^{15}N application methods on the distribution of root amino acids and ^{15}N allocation

Results in this section address the objective (ii) set out in section 3.2.

3.4.2.1. Distribution of root amino acids

A consistent pattern of AA in the roots was found between different treatments (Figure 3.6), showing that the application method did not affect the relative distribution of the AAs in the roots of white clover. Generally, the leaf treatment was shown to have the lowest concentration of individual AAs, especially compared to the control. However, no significant difference was found between the total hydrolysable AA content for each different treatment (Table 3.7). Asx was shown to be the most concentrated AA in the roots.

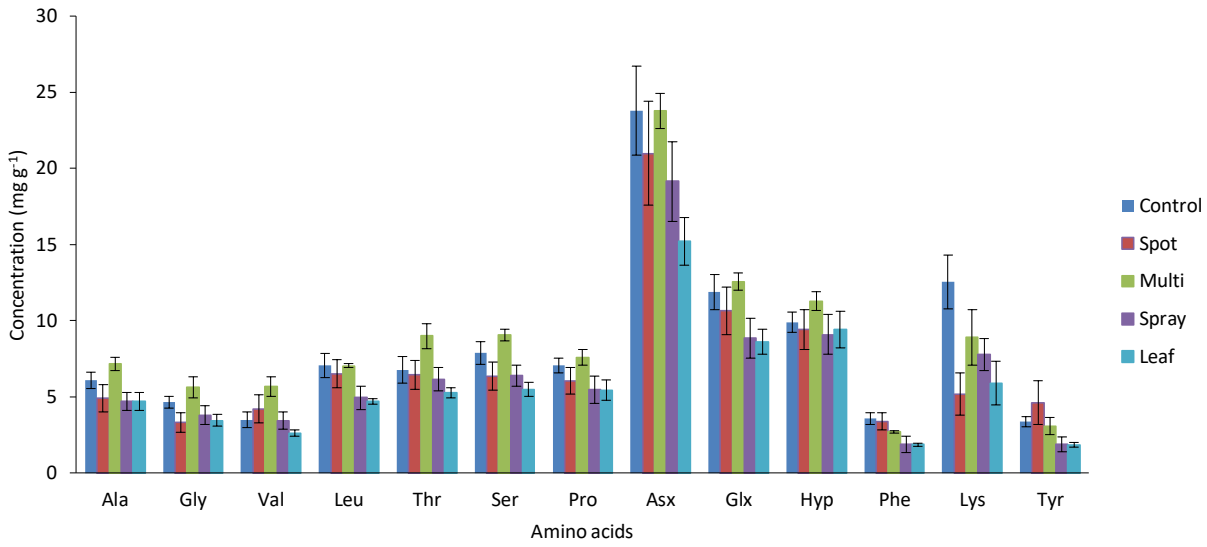


Figure 3.6. Concentration of AAs [mg of AA per gram of root (mg g^{-1})] in the roots of white clover (*Trifolium repens*) plants growing in rhizotrons following five different application methods of applying $^{15}NH_4{}^{15}NO_3$ (1 mM at 10 atom %) (mean ± standard error; n=4)

Similarly, the different treatments were not found to affect the total N and C contents of the roots (Table 3.7), with the root total N varying between 2.24 to 2.52% and root total C between 36.3 to 40.6% for all treatments.

Table 3.7. Mean root total N (% TN), root total C (%TC), total root hydrolysable AA content and total root hydrolysable content which is N (mg g^{-1}) for white clover roots (*Trifolium repens*) following the application of $^{15}NH_4{}^{15}NO_3$ (1 mM at 10 atom %) through five different methods. One-way AVONA result comparing the effect of different application methods on the %TN, %TC, total root hydrolysable AA content and total root hydrolysable content which is N in the soil.

	% TN	% TC	Total hydrolysable amino acid (mg g^{-1})	Total hydrolysable amino acid N (mg g^{-1})
Control	2.38	37.0	108.1	13.8
Spot	2.35	38.8	92.1	11.2
Multi	2.23	36.8	113.5	14.3
Spray	2.35	37.2	83.6	10.6
Leaf	2.36	38.7	74.5	9.3
ANOVA	NS	NS	NS	NS

NS: main effect or interaction not significant at the P<0.05 level.

3.4.2.2. ^{15}N allocation to amino acids in the roots

The application method did not affect the distribution of ^{15}N into the AAs in the roots (Figure 3.7), showing a general increase in $\delta^{15}N$ values in the AAs with increased bulk ^{15}N values (Figure 3.4). Therefore, the application method did not affect the physiological routing of the AAs. Phe was shown to have the greatest $\delta^{15}N$ value enrichment (even in the control it was the most enriched AA).

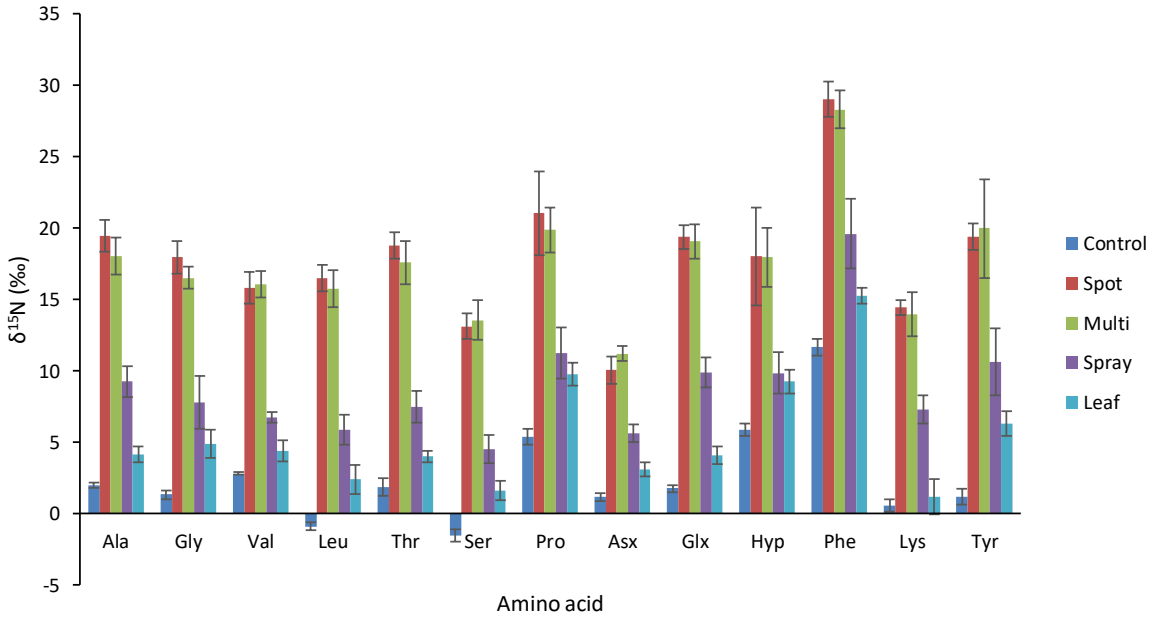


Figure 3.7. $\delta^{15}N$ values of the AAs in the roots of white clover (*Trifolium repens*) plants growing in rhizotrons after $^{15}NH_4{}^{15}NO_3$ (1 mM at 10 atom %) application through different techniques. (mean ± standard error; n= 4)

From these results, the increase in $\delta^{15}N$ values of individual AA can be used to determine the percentage of ^{15}N applied at time 0 incorporated into each AA. These calculations are able to reflect the concentration and the $\delta^{15}N$ value of the AA allowing the subtle changes (increase compared to the control) in the incorporation of ^{15}N into AAs to be identified which can not been seen in the figures alone (Figure 3.7).

Results showed a lower percentage incorporation of the applied ^{15}N-label into individual AAs in the roots (Table 3.8) than the bulk percentage incorporations (Table 3.6), suggesting that not all N taken up by the plant had been converted into AAs and proteins. Unsurprisingly, the spot and the multi treatment showed the largest incorporation, followed by the spray and then leaf treatments. For the spray and leaf treatments, Ser and Glx were shown to have no incorporation

of ^{15}N-label, which cannot be seen from Figure 3.7. There appeared to be large variation in the percentage incorporation into different AAs with different treatments, which cannot be seen in Figure 3.7. For example, Lys was shown to have a higher percentage incorporation than Asx for the multi treatment (2.52 to 2.44% respectively), while Lys was greatest in the spray treatment, and in the spot and leaf treatment Hyp showed the highest incorporation. Generally, these three AAs, along with Thr, had the greatest incorporation values.

Table 3.8. Incorporation of the applied ^{15}N-label in the bulk roots incorporated into individual AAs (%) in white clover (*Trifolium repens*) plants growing in rhizotrons after $^{15}NH_4{}^{15}NO_3$ (1 mM at 10 atom %) application through different techniques. Maximum values for each treatment are highlighted in bold.

	Spot	Multi	Spray	Leaf
Alanine	1.23	1.87	0.65	0.16
Glycine	0.94	1.65	0.51	0.23
Valine	0.61	0.93	0.20	0.05
Leucine	1.14	1.28	0.39	0.17
Threonine	1.44	2.10	0.79	0.34
Serine	0.62	1.05	-	-
Proline	1.45	1.79	0.76	0.56
Aspartic acid	1.70	2.44	0.86	0.21
Glutamic acid	0.76	0.94	-	-
Hydroxyproline	**1.78**	2.17	1.05	**0.89**
Phenylalanine	0.75	0.63	0.27	0.22
Lysine	1.28	**2.52**	**1.29**	0.18
Tyrosine	0.62	0.49	0.15	0.09

3.4.3. Uptake and distribution of ^{15}N over time *via* the leaf-labelling technique in rhizotrons

Results in this section address the objective (iii) set out in section 3.2.

3.4.3.1. Partitioning of ^{15}N into plant parts and soil over time

The results showed that repeating the leaf-labelling method achieved higher enrichment levels (Figure 3.8) than in the initial experiment comparing ^{15}N-labelling methods (Figure 3.3). Here whole rhizotrons were sampled at each time point showing how ^{15}N enrichment changed over-time with the application of ^{15}N-label through leaf submersion. Overall, results showed a very similar pattern to before, with $\delta^{15}N$ values of plant parts increasing up to 100 h before decreasing after the labelling source was removed at 72 h, although it was unclear why $\delta^{15}N$ values were then seen to increase at 361 hours. No significant difference was found between the interaction of time after labelling and different plant parts (time*plant part $P>0.05$). However, a significant difference was found between different time sampling points ($F_{10, 117}= 5.673$, $P= 0.000$), where 0 hours (time before application of label) was found to differ from 100 and 361 hours. Although generally no significant difference was found between the majority of time points, showing very little uptake over-time. However, no difference was also found between the $\delta^{15}N$ values of different plant parts, further showing a uniform distribution of ^{15}N-label throughout the plants as in the previous experiment.

Results showed very little change in the soil $\delta^{15}N$ values (Figure 3.8), ranging between 4.7 and 6.8‰. A slight increase in values was seen at 54 h, however, no significant difference was found over-time. This would suggest very low potential for seeing N-transfer between plants, as it is likely that $\delta^{15}N$ values would need to be a lot higher to enrich the whole plant-soil system.

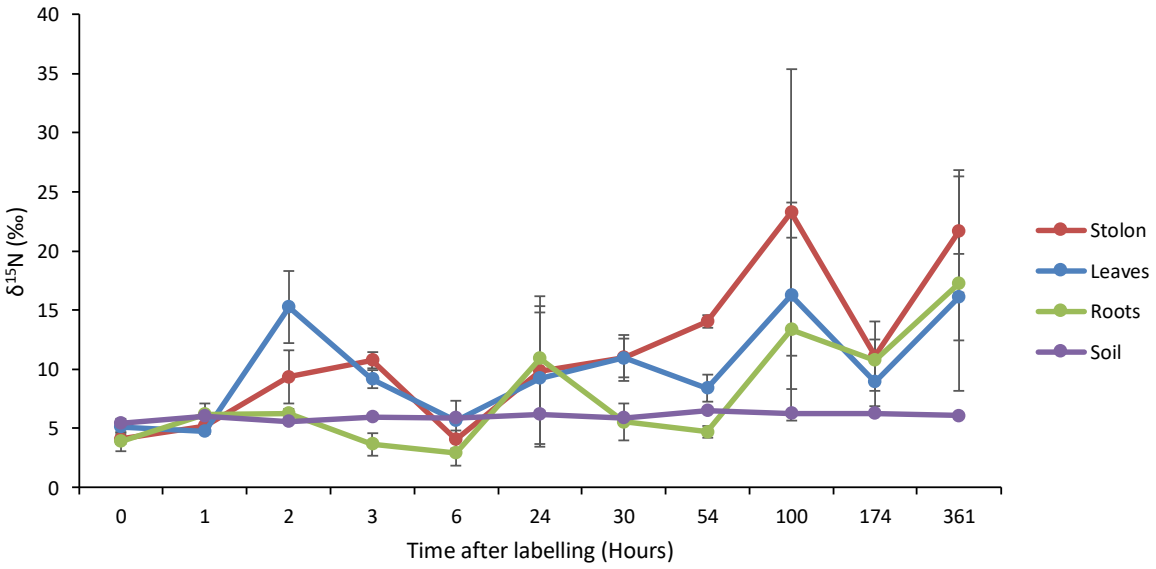


Figure 3.8. Temporal changes in $\delta^{15}N$ values for the leaves, stolon, roots of white clover (*Trifolium repens*) plants and soil growing in rhizotrons after leaf-labelling with 30 mM $^{15}NH_4{}^{15}NO_3$ at 10 atom % (with outliers removed, as described in Section 2.5.8). (mean ± standard error; n=3 or 4)

Unsurprisingly from looking at the bulk $\delta^{15}N$ values, very little incorporation of the ^{15}N-label applied to the leaves was found to be incorporated into different plants parts and the soil (Figure 3.9). Interestingly, the soil was shown to have the greatest incorporation, although at maximum this was only 2.7%. Even the leaves where the label was applied were shown to have surprisingly low incorporation, reaching a maximum of 1.2% at 361 h.

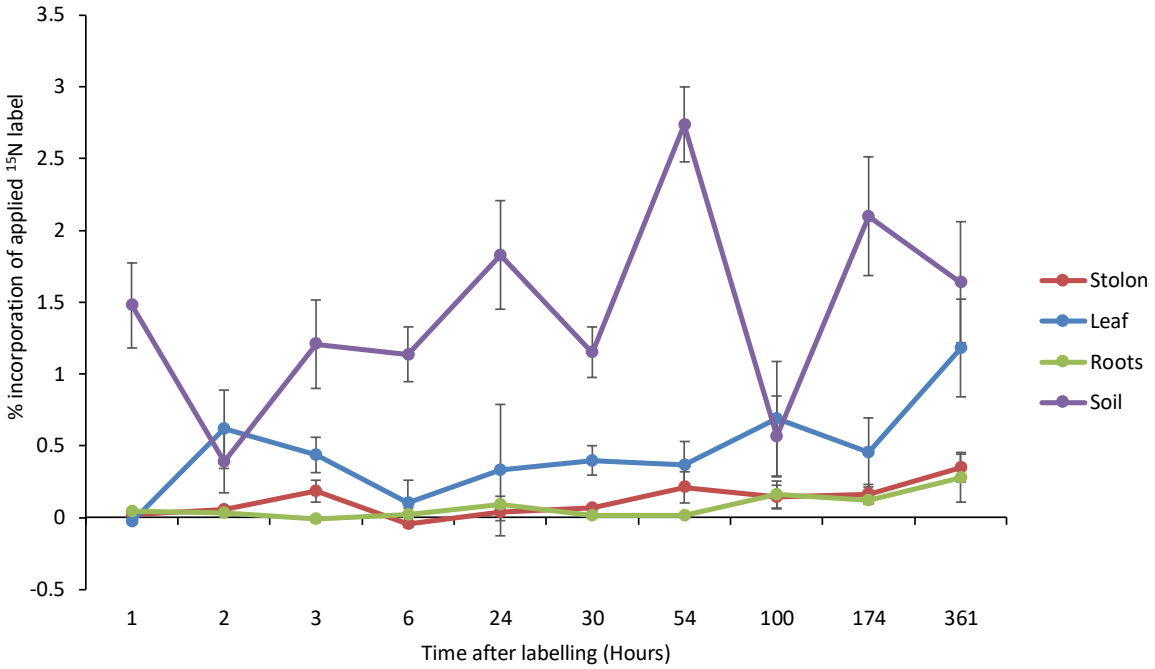


Figure 3.9. Percentage incorporation of the applied ^{15}N-label (30 mM $^{15}NH_4{}^{15}NO_3$ at 10 atom %) to white clover leaves (*Trifolium repens*) incorporated into different plant parts and the soil (with outliers removed, as described in Section 2.5.8). (mean ± standard error; n=3 or 4)

Furthermore, the relatively low δ^{15}N values achieved in the verification of the leaf-labelling technique experiment were surprising with the increased ^{15}N concentration used and less biomass produced to dilute the ^{15}N-label within plant parts. Approximately one-quarter of the biomass produced in the comparing ^{15}N-labelling methods experiment was produced in the verification of the leaf-labelling technique experiment, which looked at the temporal changes in ^{15}N uptake over time for all plants parts. The plant biomass produced by each plant varied between 230 to 1050 mg (Table 3.9). Despite sampling over-time, no difference was found in the total plant biomass produced at each sampling time, showing that plants didn't significantly grow over a 361 h study. Similarly, no difference was found in the N content of the stolon, leaves or roots over the time course of the experiment.

Table 3.9. Dry matter and N-content for white clover (*Trifolium repens*) plant parts sampled during a 361 h leaf labelling study with $^{15}NH_4^{15}NO_3$ (30 mM at 10 atom %), (mean ± standard error; n=4). One-way ANOVA result comparing the effect of time on the resultant plant dry matter and N content.

	Dry matter (mg plant^{-1})				N content (mg plant^{-1})		
	Stolon	Leaves	Roots	Total	Stolon	Leaves	Roots
0	77.7 ± 15.7	298 ± 58.6	91.6 ± 20.6	467 ± 90.3	2.6 ± 0.7	13.4 ± 3.0	3.1 ± 0.7
1	156 ± 37.9	361 ± 78.4	144 ± 35.4	660 ± 141	5.7 ± 1.2	16.4 ± 3.3	5.3 ± 1.1
2	96.5 ± 27.7	342 ± 98.1	146 ± 65.4	584 ± 184	3.1 ± 1.0	15.0 ± 4.2	4.3 ± 1.6
3	131 ± 25.9	430 ± 116	135.9 ± 40.7	698 ± 174	5.2 ± 1.5	18.8 ± 4.6	4.7 ± 1.4
6	313 ± 136	308 ± 84.2	153 ± 39.7	774 ± 93.1	11.3 ± 5.1	14.6 ± 3.8	5.1 ± 1.0
24	101 ± 26.5	355 ± 57.3	122 ± 41.0	578 ± 45.1	3.3 ± 1.2	15.9 ± 2.3	4.7 ± 1.6
30	85.1 ± 19.8	355 ± 44.8	85.5 ± 12.0	525 ± 75.8	2.7 ± 0.7	16.1 ± 1.8	3.3 ± 0.4
54	129 ± 19.0	470 ± 35.3	152 ± 20.4	752 ± 53.2	4.7 ± 0.6	22.6 ± 2.1	5.6 ± 0.6
100	78.0 ± 13.2	338 ± 35.3	92.3 ± 18.4	508 ± 102	2.6 ± 0.7	15.8 ± 3.6	3.7 ± 0.7
173	124 ± 27.2	478 ± 70.5	137 ± 24.0	739 ± 144	4.4 ± 1.0	22.0 ± 4.3	5.0 ± 0.8
361	155 ± 33.0	433 ± 105	128 ± 33.0	676 ± 170	3.7 ± 1.1	20.1 ± 4.4	4.7 ± 1.1
ANOVA	NS	NS	NS	NS	NS	NS	NS

NS: main effect or interaction not significant at the P<0.05 level.

3.4.3.2. Distribution of amino acids in plant parts and soil over-time

Each different plant part studied exhibited a different distribution of AAs (Figure 3.10). The stolon had a high concentration of Asx, this is unsurprising due to the important role that stolon play in nutrient transport in clover and the fact that in clover Asp is the major transport molecule for N (Paynel et al., 2001a). However, for the leaves slightly more Glx was found than Asx, with a smaller difference in concentration of other AAs. The distribution of AAs in the roots differed from the comparing ^{15}N-labelling technique experiment (Figure 3.6), where Asx was the major AA. Findings in Figure 3.10 were not so clear cut, with fairly equal concentrations of most AAs, and the majority of AAs varying with time and Asx being the predominant AA at 24 and 100 h but Glx at 0 and 361 h. For the plant parts (stolon, leaves and roots) the majority of AAs exhibited a concentration increase with time, especially when comparing the sampling at 0 hours, where no ^{15}N-label had been applied, to 361 h. When comparing the total hydrolysable AA content of different plant parts (Table 3.10), a significant difference was found between different sampling time points for the stolon ($F_{3,11}$= 9.768, P=0.002), where the total hydrolysable AA content at time 0 was found to be significantly different and lower than the other three sampling points, however, no difference was found between the three time points where ^{15}N was applied to the plant. For the leaves, a significant difference in the total hydrolysable AA content was found ($F_{3,12}$=4.442, P=0.026), however, only a difference between time 0 and 361 h was evident. For the roots, a significant difference was found ($F_{3,11}$= 4.055, P=0.036), however, this time the difference was between time 0 and 100 h. The total N and C contents were seen to vary between different plant parts, with the leaves having the highest N content with C being much more evenly distributed between the different plant parts (Table 3.10). No significant difference was found between the N content of different plant parts over-time, and for the C content no difference was found for the stolon and leaves over-time. However, for the roots, a significant difference was found ($F_{3,12}$ =11.759, P=0.001), where the C content at time 0 was found to be lower than 100 and 361 hours but not at 24 hours.

For the soil, the major AAs present were Ala, Asx, Glx and Gly (Figure 3.10d). There was no visible trend shown with individual AA concentrations and time, and similarly no significant difference was found between time and total hydrolysable AA content (Table 3.10). Similarly, the total N and C contents did not significantly differ over-time, varying between 0.49 and 0.58% for N and 4.61 and 5.30% for C.

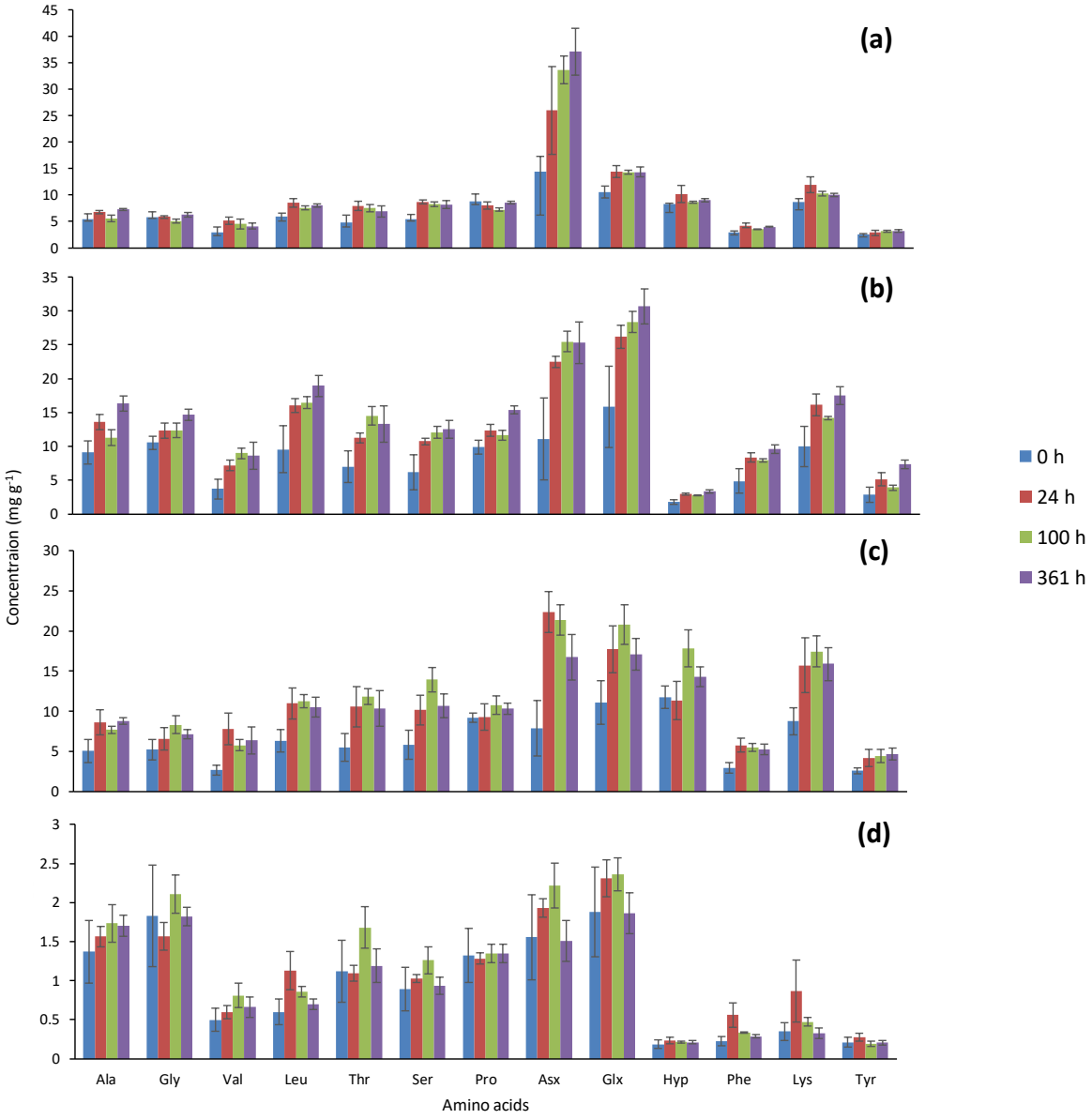


Figure 3.10. Concentration of AAs [mg of AA per gram of sample (mg g^{-1})] over time in (a) stolon, (b) leaves, (c) roots, and (d) soil of white clover (*Trifolium repens*) plants growing in rhizotrons following the application of $^{15}NH_4{}^{15}NO_3$ (30 mM at 10 atom %) through the leaf-labelling technique. (mean ± standard error; n=3 or 4)

Table 3.10. Mean total N (% TN), total C (%TC), total hydrolysable AA content and total hydrolysable content which is N (mg g^{-1}) for different plant parts in white clover (*Trifolium repens*) and soil following the application of $^{15}NH_4{}^{15}NO_3$ (30 mM at 10 atom %) through the leaf-labelling technique. One-way ANOVA result comparing the effect of time on the %TN, %TC and total hydrolysable amino acid content in different plant parts and soil.

	% TN	% TC	Total hydrolysable amino acid (mg g^{-1})	Total hydrolysable amino acid N (mg g^{-1})
Stolon				
0 h	3.22	39.3	87.6	11.1
24 h	3.22	38.9	121.7	15.3
100 h	3.17	41.8	119.3	15.0
361 h	3.15	41.0	126.8	16.0
ANOVA	NS	NS	P=0.002	-
Leaves				
0 h	4.36	38.5	102.3	13.3
24 h	4.52	39.4	164.8	21.1
100 h	4.64	39.1	169.9	21.5
361 h	4.78	40.1	193.6	24.6
ANOVA	NS	NS	P=0.026	-
Roots				
0 h	3.43	33.0	85.6	10.7
24 h	3.67	33.0	141.0	17.9
100 h	4.00	40.8	157.0	19.9
361 h	3.65	40.1	138.0	17.6
ANOVA	NS	P=0.001	P=0.036	-
Soil				
0 h	0.51	4.7	12.0	1.58
24 h	0.53	5.0	14.4	1.87
100 h	0.55	5.1	15.6	2.04
361 h	0.53	5.0	12.7	1.68
ANOVA	NS	NS	NS	-

NS: main effect or interaction not significant at the P<0.05 level.

3.4.3.3. ^{15}N uptake into individual plant parts and soil amino acids over-time

Plant parts showed a general trend of increasing δ^{15}N values of individual AAs with time (Figure 3.11), with AAs in the stolon showing the greatest enrichment. The ^{15}N-label was shown to be equally distributed between the AAs, with no clear trend of one AA being more enriched than another. However, AA δ^{15}N values of different plant parts tended to be more variable than the bulk δ^{15}N values (Figure 3.8), making it difficult to spot trends in results and comment on the incorporation of ^{15}N-label into AAs. For the soil, AA δ^{15}N values were much more consistent, however, no ^{15}N enrichment was shown over-time, which parallels the findings for the bulk δ^{15}N value results (Figure 3.8). Despite this, the bulk soil represented the largest incorporation of the ^{15}N-label (Figure 3.9), however, this was due to the calculation taking into account the mass of the sample (100 g of soil in each rhizotron) compared to the smaller mass of plant biomass produced in each rhizotron (Table 3.9)

For plant parts which were sampled before the ^{15}N-label was applied (0 hours), Phe was shown to be the most ^{15}N enriched in the stolon, while Tyr and Phe were shown to be the most ^{15}N enriched in the leaves and roots. Again, the AAs were seen to display a different pattern than Figure 3.7, which showed that Phe undoubtedly was the most ^{15}N enriched AA in the roots, whether or not ^{15}N was applied to the root system.

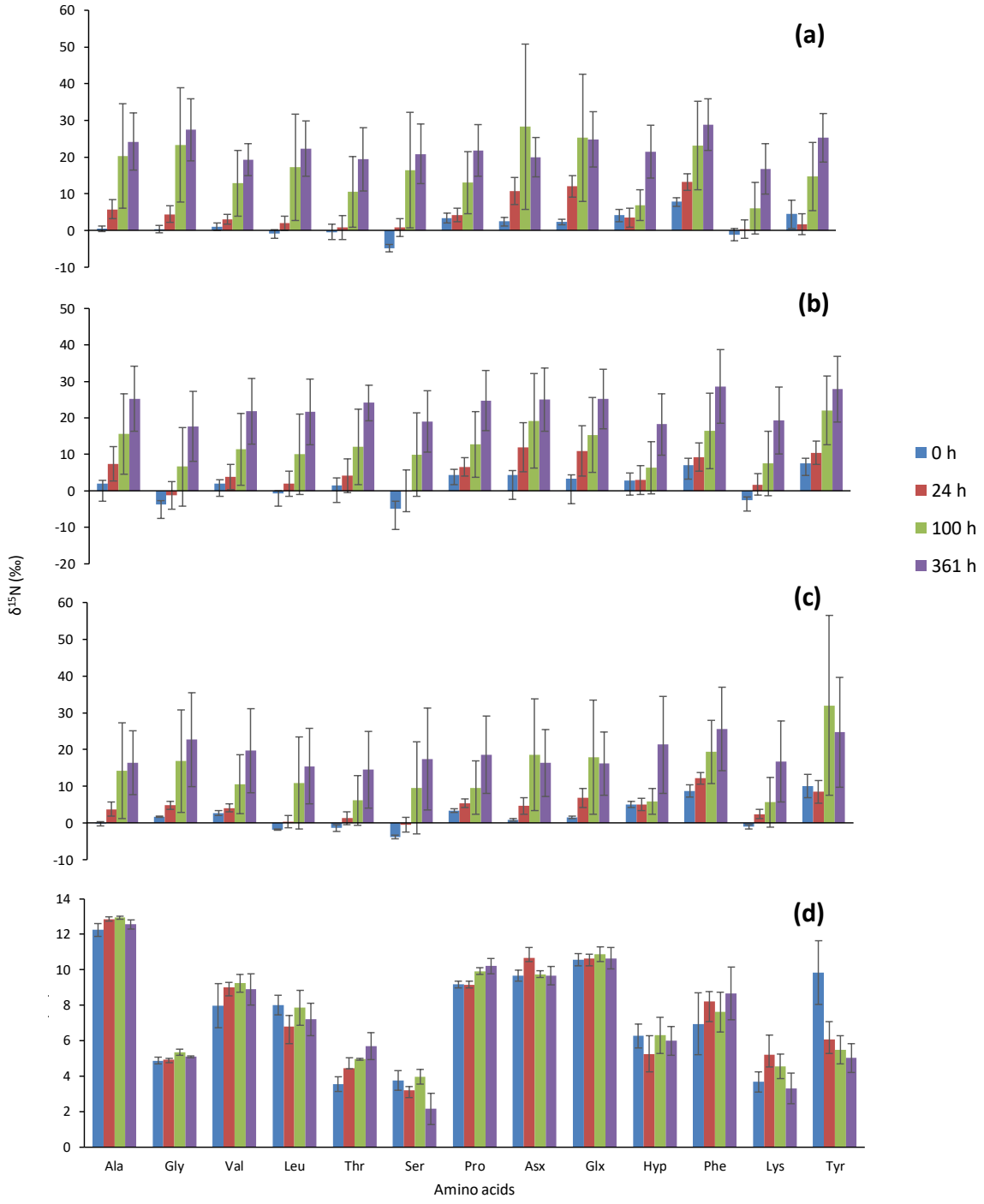


Figure 3.11. $\delta^{15}N$ values of the AAs in (a) stolon, (b) leaves, (c) roots and (d) soil of white clover (*Trifolium repens*) plants growing in rhizotrons following the application of $^{15}NH_4{}^{15}NO_3$ (30 mM at 10 atom %) through the leaf-labelling technique (mean ± standard error; n=3 or 4, minus outliers identified in bulk $\delta^{15}N$ values)

Due to the complexity of $\delta^{15}N$ values of individual AAs shown in Figure 3.11 and clear trends not being identifiable, calculating the percentage incorporation of the applied ^{15}N-label into individual AAs helped to clarify the findings (Figure 3.12). This allowed subtle changes (increase compared to the control) in the incorporation of ^{15}N into the AAs to be identified, as it takes into account the $\delta^{15}N$ values of individual AA as well as the concentration. For the stolon at 100 and 361 hours, the majority of AAs showed a 0.005-0.02% incorporation of the applied ^{15}N-label. However, Asx was shown to have the largest incorporation of 0.045%. Lower incorporations were shown at 24 h for the stolon, showing the importance of time when using the leaf-labelling technique, as this is a slower method of uptake of ^{15}N.

For the leaves, higher percentage incorporation into AAs was seen, with a much more defined trend of increased incorporation with time (Figure 3.12 b). However, for the leaves there was no clear pattern in percentage incorporation into individual AAs, with a fairly equal distribution being shown. However, results showed a negligible to very minimal incorporation into Hyp, Phe and Tyr.

A similar pattern to the leaves was also seen in the roots, however, the incorporation was lower. This is unsurprising as the ^{15}N-label was applied to the leaves and not the roots, requiring transport down through the plant. Again, the lowest incorporation was at 24 h, with samples at 100 and 361 h being less distinguishable in percentage incorporation. Additionally, the leaves and roots both showed a large amount of incorporation into Lys at 361 h.

For the soil, incorporation of the applied ^{15}N-label was variable between the AAs. Not all AAs were found to incorporate the applied ^{15}N-label, no incorporation at any time point was found for Tyr, with Leu and Hyp showing some incorporation at 100 h. For the soil, no clear pattern was seen with time, with 100 h generally having the highest incorporation into a range of AAs, especially for Gly. The lower incorporation of the applied ^{15}N-label into the AAs compared to the bulk soil (Figure 3.9), showed that the ^{15}N-label was incorporated into other N containing compounds (inorganic N, such as NO_3^- and NH_4^+ or into other components of the organic soil, such as amino sugars). Likewise, the ^{15}N-label in the soil could have been re-taken up by the plant or used by microbes, producing lower incorporation into the AAs than seen with the bulk soil.

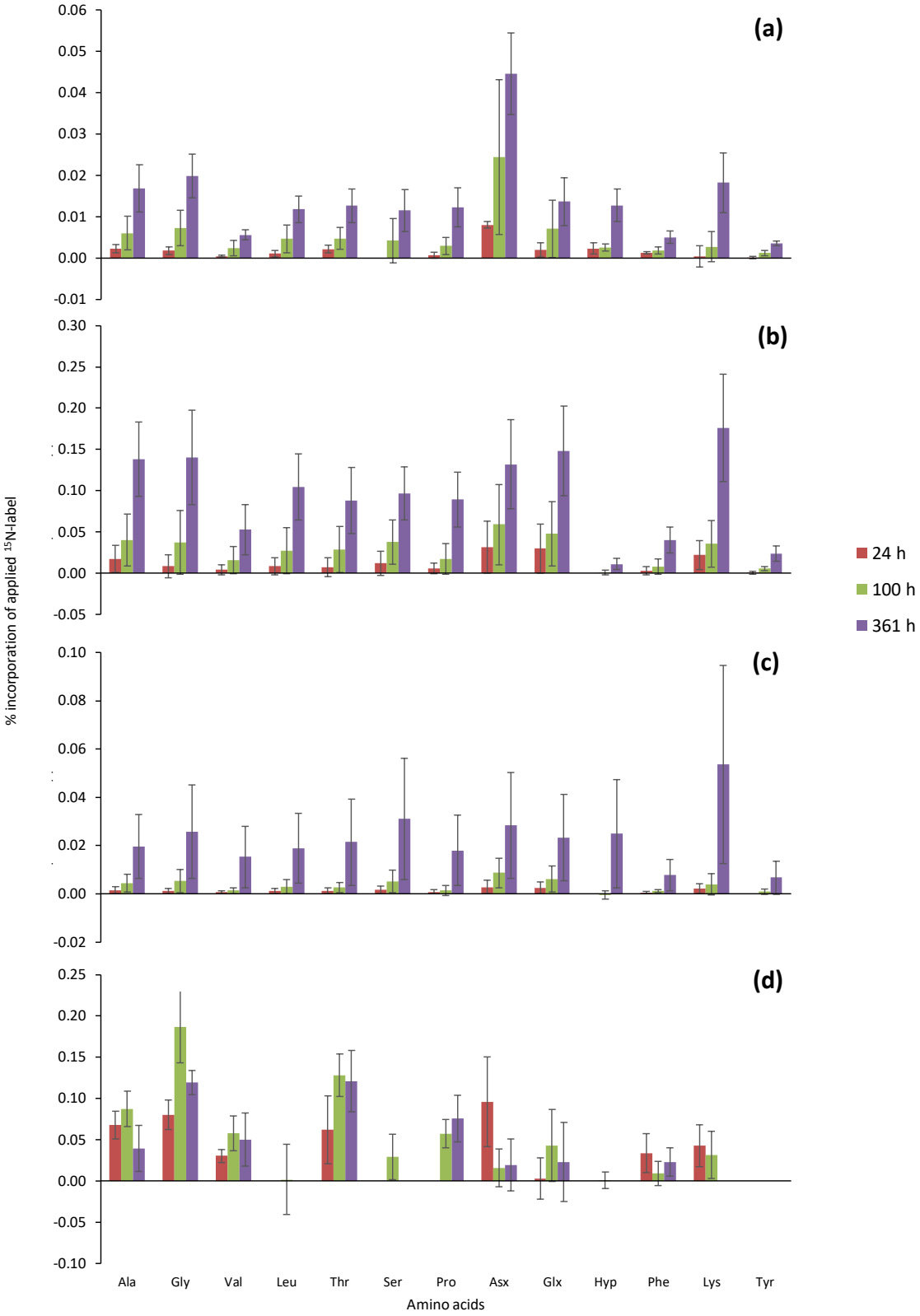


Figure 3.12. Incorporation of the applied ^{15}N-label (30 mM $^{15}NH_4{}^{15}NO_3$ at 10 atom %) through the leaf-labelling technique into individual AAs (%) in the different plant parts of white clover (*Trifolium repens*) (a) stolon, (b) leaves, (c) roots and (d) soil in rhizotrons over-time. (mean ± standard error; n=3 or 4, minus outliers identified in bulk δ^{15}N values.)

3.4.4. Potential for ^{15}N uptake *via* the leaf-labelling study by eliminating background nitrogen

Results in this section address the objectives (iv) and (v) set out in section 3.2, showing that all plant parts were vastly more enriched with ^{15}N (Figure 3.13) when labelling with 30 mM $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ at 98 atom % and growing within sand than the previous two experiments (Figures 3.3, 3.4, 3.8). Labelling with $CO(^{15}NH_2)_2$ resulted in the ^{15}N enrichment of plant parts being approximately three times greater than that of $^{15}NH_4{}^{15}NO_3$ for the stolon and leaves, and twice that observed in the roots. When comparing the interaction between plant parts and treatments *via* a two-way ANOVA, no significant difference was found between plant parts, showing a uniform distribution of the ^{15}N-label within the plants (Table 3.11). However, as expected, a significant difference was found between treatments ($F_{41,2}$= 29.850, P= 0.000), where the $CO(^{15}NH_2)_2$ was found to differ and be higher than the $^{15}NH_4{}^{15}NO_3$ and the control.

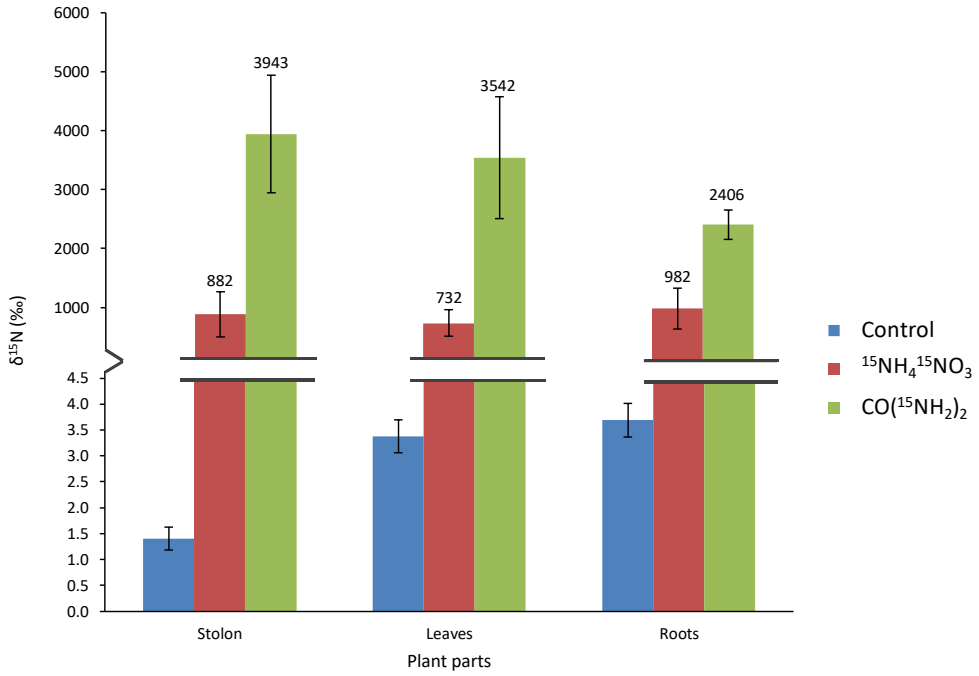


Figure 3.13. $\delta^{15}N$ values of different plant parts in each treatment for white clover (*Trifolium repens*) plants growing in sand within rhizotrons, plants either received DDW for the control, $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ (30mM at 98 atom %) through the leaf-labelling technique and sampled after 100 h. (mean ± standard error; n=5 or 6, with outliers removed as described in Section 2.5.8)

Table 3.11. Statistical results for experiment looking at the potential for ^{15}N uptake via the leaf-labelling technique by eliminating background nitrogen.

Statistical test	Interaction	P-value
Two-way ANOVA	Plant part * Treatment	NS
	Plant part	NS
	Treatment	P=0.000

NS: main effect or interaction not significant at the P<0.05 level.

Despite achieving greater ^{15}N enrichment in this experiment by eliminating plant uptake of N (by using sand as a growth substrate, although N_2-fixation was still possible) and increasing the atom % used, it remained the case that a low incorporation of the applied ^{15}N-label into bulk $\delta^{15}N$ values was still observed (Table 3.12). For the $^{15}NH_4{}^{15}NO_3$ applied to the leaves, there was less than 1% incorporation into the bulk $\delta^{15}N$ values of all plant parts, with slightly more being seen for $CO(^{15}NH_2)_2$ at 1-3%.

Growing clover plants within sand in rhizotrons was seen to reduce the biomass produced compared to previous experiments (Table 3.13 compared to Table 3.5 and 3.9). Although it should be noted that previous experiments used two clover plants within rhizotrons, so it would be expected that the biomass produced would be approximately half. Taking this into consideration, approximately 10 times more total biomass was produced in the comparing ^{15}N labelling methods experiment (Table 3.5) than this experiment eliminating background N (Table 3.13), and twice as much in the verification of the leaf-labelling technique experiment (Table 3.9). From this it can be concluded that the sand reduced growth compared with the soil. Furthermore, it proved difficult to establish plants within sand in this experiment, requiring more replicates to ensure that results were obtained.

For the plants growing in sand, plants produced an average total biomass of all treatments of 181 mg, furthermore, no significant difference was found between the total biomass produced by each treatment. However, for this experiment, a significant difference was found between treatments and the total N content of the leaves and roots. The application of $^{15}NH_4{}^{15}NO_3$ and $CO(^{15}NH_2)_2$ increased the N content in the plant compared to the control (Table 3.13). The N content was seen to vary between 1.65-3.68% in the leaves, 0.81-2.15% in the stolon and 1.57-2.7% in the roots. However, the C content of each plant part was not affected, with an average of 42% in the leaves, 39% in the stolon and 34% in the roots.

Table 3.12. Percentage incorporation of applied ^{15}N-label NH_4NO_3 or $CO(NH_2)_2$ (30mM at 98 atom%) via the leaf-labelling technique into bulk plant δ^{15}N values (%) of white clover (*Trifolium repens*). (mean ± SEM)

	Stolon	Leaves	Root
$^{15}NH_4{}^{15}NO_3$	0.28 ± 0.11	0.87 ±0.29	0.78 ± 0.25
$CO(^{15}NH_2)_2$	1.29 ± 0.47	3.40 ± 1.46	1.31 ± 0.27

Table 3.13. Dry matter and N-content for white clover (*Trifolium repens*) plant parts sampled during a 100 h leaf-labelling study with application of $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ or DDW water for the control (mean ± standard error; n=4). One-way ANOVA result comparing the effect of different labelling substrates on the resultant dry matter and plant N content.

	Dry matter (mg plant^{-1})				N content (mg plant^{-1})		
	Stolon	Leaves	Roots	Total	Stolon	Leaves	Roots
Control	22.6 ± 3.7	45.8 ± 5.4	37.0 ± 7.2	105.4 ± 47.9	0.24 ± 0.03	0.91 ± 0.08	0.65 ± 0.12
$^{15}NH_4{}^{15}NO_3$	47.0 ± 12.5	97.5 ± 14.0	96.5 ± 22.4	241.0 ± 47.9	0.89 ± 0.25	3.00 ± 0.38	2.12 ± 0.37
$CO(^{15}NH_2)_2$	39.6 ±9.9	77.9 ± 20.4	73.0 ± 18.2	196.4 ± 46.9	0.73 ± 0.20	2.30 ± 0.65	1.56 ± 0.34
ANOVA	NS	NS	NS	NS	NS	P=0.009	P=0.011

NS: main effect or interaction not significant at the P<0.05 level.

For the clover plants growing in sand in the rhizotrons, clover exudation was investigated [addressing objective (vi)], however, due to the low growth rate of the plants, no evidence of exudation of AAs into the sand could be found. The bulk sand was analysed for total C and N content, finding no evidence of N input into the sand growth medium, and only a very small amount of C in half of samples (0.001 to 0.006%).

3.4.5. Amino acid recovery from sand

Results in this section address the objective (vii) set out in section 3.2. AA recovery from sand was seen to vary between different AAs as shown in the chromatogram in Figure 3.14 and percentage loss in Figure 3.15. Not all AAs were shown to be affected, most noticeably the greatest losses were exhibited by Lys.

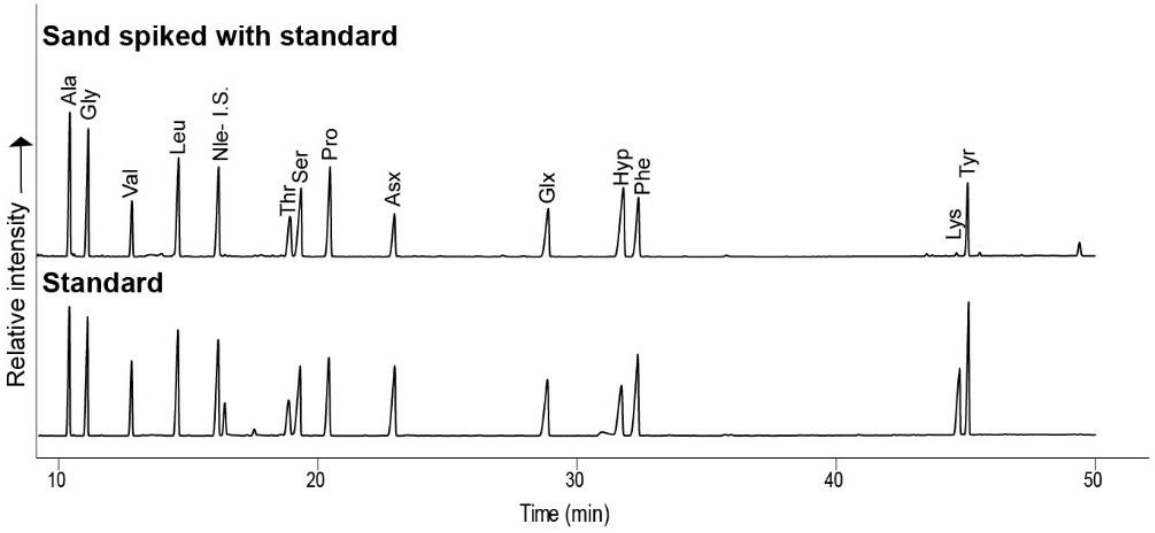


Figure 3.14. Typical GC-FID chromatogram of *N-acetyl-O*-isopropyl derivatised AA standard and recovery of AA standard in sand.

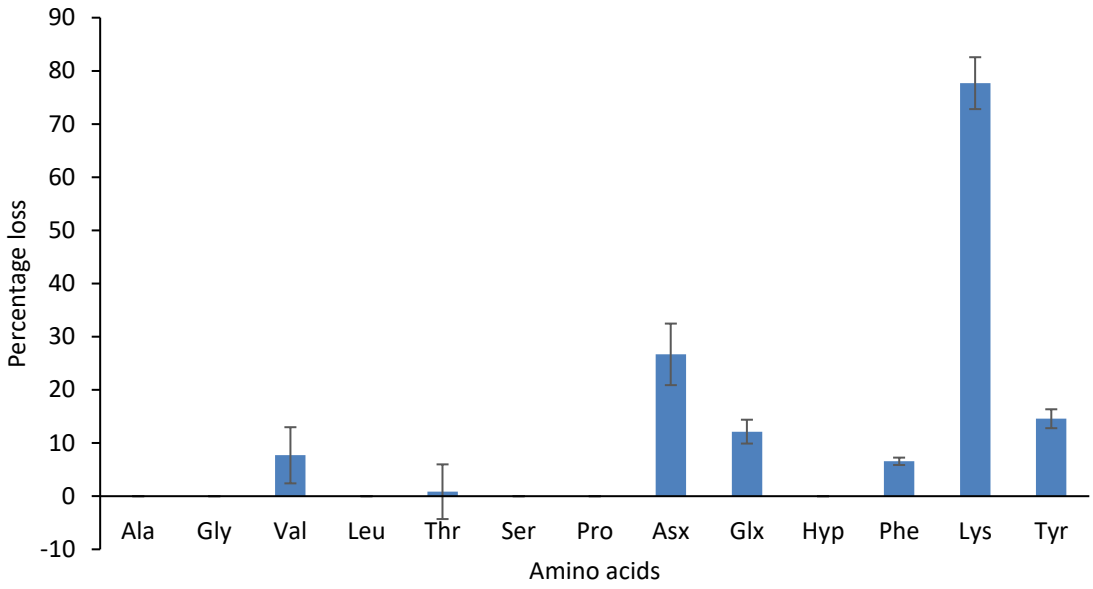


Figure 3.15. Percentage loss of AAs from sand spiked with AA standard (%) (mean ± standard error; n=6)

3.4.6. Determining the suitability of the split-root labelling technique

Results in this section address the objectives (v), (vi), (vii), (iv) and (ix) set out in section 3.2.

3.4.6.1. Clover uptake of ^{15}N *via* the split-root labelling technique

The split-root labelling technique was shown to be effective for substantially enriching all plant parts with ^{15}N, even resulting in some enrichment in the soil compared to the control (Figure 3.16). The use of the split-root technique was seen to increase the $\delta^{15}N$ values in the plant part receiving the ^{15}N-label by over 10 times that of values achieved using the leaf-labelling technique in sand (Figure 3.13). However, the $\delta^{15}N$ value of different plant parts were seen to decline considerably as transport distance from the labelling source increased, with ^{15}N being taken up and redistributed throughout the plant, showing a non-uniform distribution of the label throughout the plant.

The different labelling sources used ($^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$) were shown to affect the enrichment of different plant parts. For the clover roots in the LC and TC, more ^{15}N enrichment was found with $CO(^{15}NH_2)_2$ than $^{15}NH_4{}^{15}NO_3$, however, this difference was not significant. However, in the clover shoots, more ^{15}N enrichment was found with $^{15}NH_4{}^{15}NO_3$ than $CO(^{15}NH_2)_2$, with a significant difference observed between the two treatments ($F_{1,7}$= 9.085, P=0.020). However, the most important finding was that more ^{15}N enrichment in the soil was

found with $CO(^{15}NH_2)_2$ than $^{15}NH_4{}^{15}NO_3$. On comparison with the soil $\delta^{15}N$ values of the control, a significant difference was found ($F_{2,12}$= 10.359, P=0.002), with $CO(^{15}NH_2)_2$ differing from $^{15}NH_4{}^{15}NO_3$ and the control, which were found to not significantly differ each other (Table 3.14). The ^{15}N enrichment in the soil, represented a transfer from the clover roots into the soil of 0.37% for $^{15}NH_4{}^{15}NO_3$ and 0.58% for $CO(^{15}NH_2)_2$.

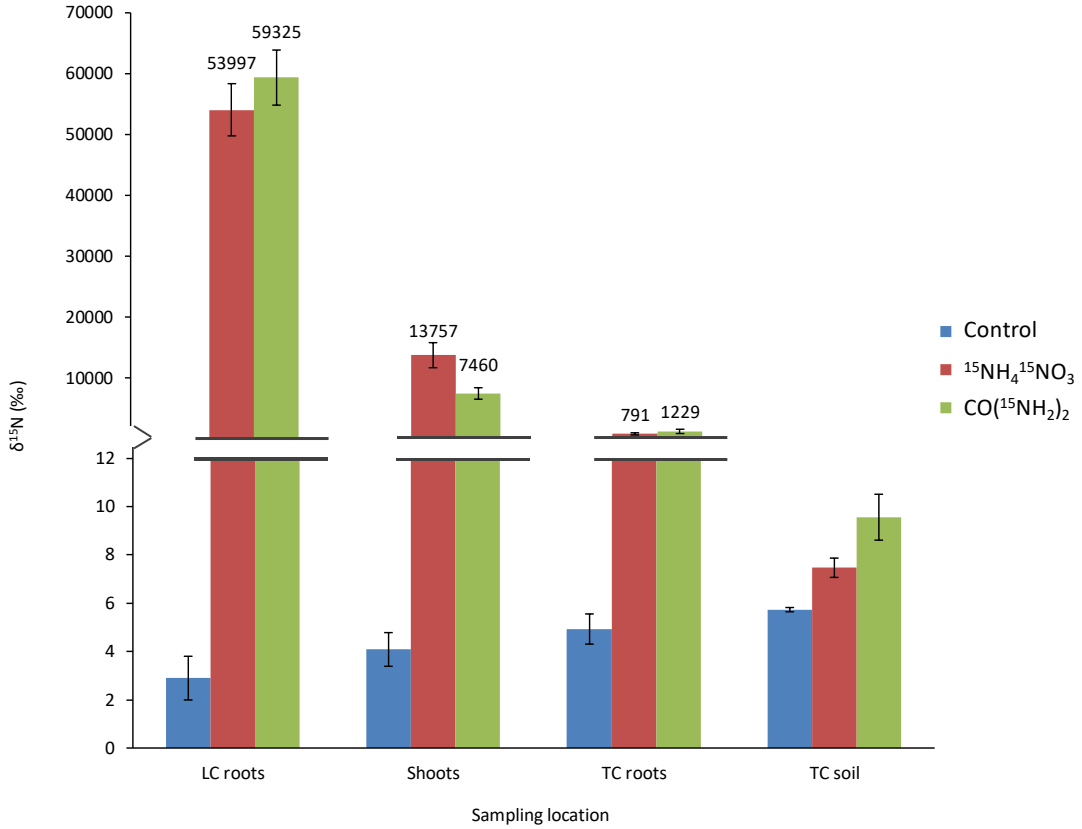


Figure 3.16. $\delta^{15}N$ values of different plant parts after application of the split-root labelling technique. White clover (*Trifolium repens*) plants were either labelled with DDW for the control, $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ (30 mM at 98 atom %) and sampled after 100h. LC- labelling compartment, and TC- transfer compartment. (mean ± standard error; n=4 or 5, with outliers removed as described in Section 2.5.8)

Table 3.14. Statistical results for split-root labelling experiment, using one-way ANOVA to compare the ^{15}N enrichment of different samples which were enriched with either $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$.

Sample	P-value
LC roots	NS
Shoots	0.020
TC roots	NS
Soil	0.002

NS: main effect or interaction not significant at the P<0.05 level.

The findings in Figure 3.16 are further supported by the percentage incorporation of the applied ^{15}N-label into the bulk $\delta^{15}N$ values of different plant parts and the soil (Table 3.15), showing a greater incorporation through the split-root labelling technique than when similar concentrations of substrates were introduced using the leaf-labelling technique (Table 3.12). For example, an incorporation of 44.7% for $^{15}NH_4{}^{15}NO_3$ and 27.5% for $CO(^{15}NH_2)_2$ was found for the shoots using the split-root labelling technique, but only 0.9% and 3.4% respectively for the leaf-labelling technique. The percentage incorporation takes into account the biomass produced by the plant, hence the shoots show a large percentage incorporation compared to the more ^{15}N enriched clover roots in the LC, due to the ^{15}N-label being incorporated into a larger amount of plant biomass (Table 3.16).

A fairly substantial amount of plant biomass was produced in the split-root labelling study (Table 3.16), compared to the verification of the leaf-labelling experiment, using two clover plants in rhizotrons (Table 3.9) (453 mg compared to 633 mg), whilst compared with eliminating background N and growing plants entirely in sand twice as much total biomass was produced (Table 3.13). There was no difference in the total biomass produced in each treatment, similarly, no difference was found in the N content of the roots in the LC or TC or the shoots.

Table 3.15. Percentage incorporation of applied ^{15}N-label $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ (30mM at 98 atom%) via the split-root labelling technique into bulk plant $\delta^{15}N$ values (%) of white clover (*Trifolium repens*) and soil. LC- labelling compartment, and TC- transfer compartment (mean ± SEM).

	Roots LC	Shoots	Roots TC	Soil
$^{15}NH_4{}^{15}NO_3$	17.2 ± 4.0	44.7 ± 6.9	0.75 ± 0.20	0.06 ± 0.01
$CO(^{15}NH_2)_2$	28.3 ± 1.4	27.5 ± 2.7	1.29 ± 0.37	0.13 ± 0.03

Table 3.16. Dry matter and N-content for white clover (*Trifolium repens*) plant parts sampled after a 100 h labelling study, where a split-root labelling technique was used to apply either DDW for the control, $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$. LC- labelling compartment, and TC- transfer compartment (mean ± standard error; n=5). One-way ANOVA result comparing the effect of labelling substrate on the resultant plant dry matter and N content.

	Dry matter (mg plant^{-1})				N content (mg plant^{-1})		
	Roots LC	Shoots	Roots TC	Total	Roots LC	Shoots	Roots TC
Control	59.6 ± 7.5	334 ± 18.0	91.2 ± 9.8	485 ± 23.0	1.16 ± 0.16	9.34 ± 0.34	2.21 ± 0.26
$^{15}NH_4{}^{15}NO_3$	41.2 ± 9.8	257 ± 12.2	82.0 ± 7.8	413 ± 34.4	0.84 ± 0.19	7.73 ± 0.24	2.06 ± 0.14
$CO(^{15}NH_2)_2$	57.6 ± 5.7	300 ± 17.8	102 ± 12.2	460 ± 27.6	1.34 ± 0.15	8.72 ± 0.59	2.53 ± 0.24
ANOVA	NS	NS	NS	NS	NS	NS	NS

NS: main effect or interaction not significant at the $P<0.05$ level.

3.4.6.2. Distribution of soil amino acids in the transfer compartment following clover growth and labelling with ^{15}N *via* the split-root labelling technique

The application of different ^{15}N treatments to the clover roots in the LC did not affect the distribution of AAs in the soil in the TC (Figure 3.17). A fairly equal concentration of most AAs was seen, ranging between 0.6 and 1.5 mg g^{-1}, the exceptions being Hyp, Phe and Tyr which were all present at much lower concentrations (<0.32 mg g^{-1}).

Interestingly, the distribution of soil AAs in this experiment slightly differed from when the AAs were studied in the verification of the leaf-labelling technique experiment (Figure 3.10d). The main difference being the concentration of Lys, which was much greater in the soil obtained from the split-root labelling technique (Figure 3.17, 0.9 to 1.5 mg g^{-1}) than previously (Figure 3.10d, 0.3 to 0.9 mg g^{-1}).

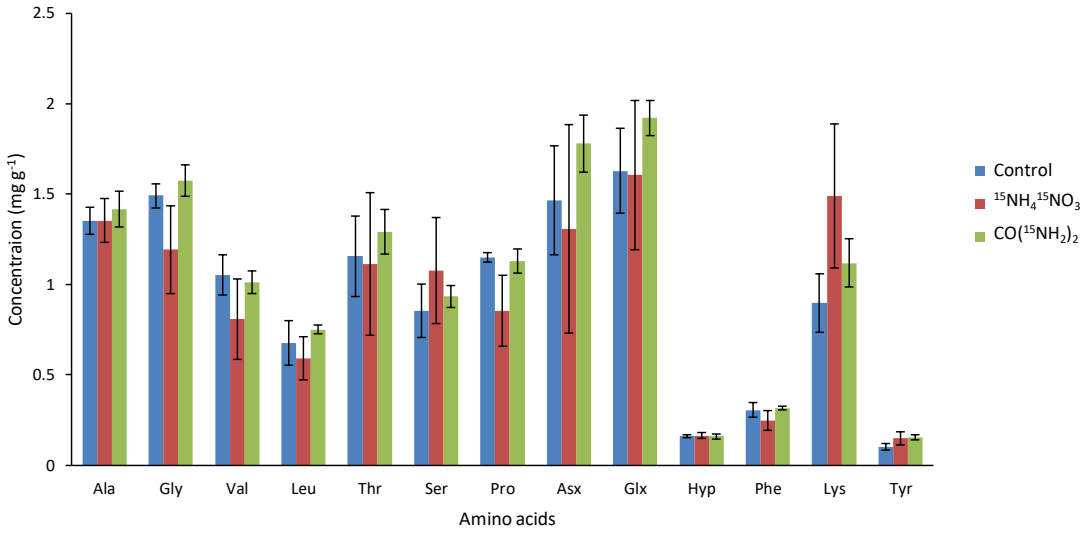


Figure 3.17. Concentration of soil AAs (TC) [mg of AA per gram of sample (mg g^{-1})] following growth of white clover (*Trifolium repens*), which was labelled through a split-root labelling technique injecting either $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ or DDW for the control in the labelling compartment. (mean ± standard error; n=5)

Furthermore, supporting the findings in Figure 3.17, different treatments applied were not seen to have a significant effect on the total hydrolysable AA content, or total N and C (Table 3.17). Similar concentrations of total hydrolysable AAs were produced in this experiment as in the verification of the leaf-labelling technique experiment (Table 3.10, 12-15.6 mg g^{-1}).

Table 3.17. Mean total N (% TN), total C (%TC), total hydrolysable AA concentrations and total hydrolysable concentrations which is in N (mg g^{-1}) for the transfer compartment soil after growth of white clover (*Trifolium repens*) which was labelled through a split-root labelling technique injecting either $^{15}NH_4{}^{15}NO_3$ or $CO(NH_2)_2$ or DDW for the control in the labelling compartment. One-way ANOVA result comparing the effect of labelling substrate on the %TN, %TC and total hydrolysable amino acid content in the soil.

	% TN	% TC	Total hydrolysable amino acid (mg g^{-1})	Total hydrolysable amino acid N (mg g^{-1})
Control	0.48	5.0	12.8	1.64
$^{15}NH_4{}^{15}NO_3$	0.50	5.0	12.0	1.62
$CO(^{15}NH_2)_2$	0.50	4.9	13.4	1.79
AVONA	NS	NS	NS	-

NS: main effect or interaction not significant at the P<0.05 level.

3.4.6.3. Allocation of ^{15}N to soil amino acids in the transfer compartment following clover growth and labelling with ^{15}N *via* the split-root labelling technique

As with the bulk $\delta^{15}N$ values for the soil in the TC, a slight enrichment was seen in the individual AAs $\delta^{15}N$ values following the application of $^{15}NH_4{}^{15}NO_3$ and $CO(^{15}NH_2)_2$ to clover plants in the LC (Figure 3.18). Again, a greater enrichment was seen with $CO(^{15}NH_2)_2$ than with $^{15}NH_4{}^{15}NO_3$ in all AAs apart from Leu. At 24‰, Pro was the most ^{15}N enriched AA for the $CO(^{15}NH_2)_2$ treatment with all other AAs ranging from 6-16‰. For $^{15}NH_4{}^{15}NO_3$, Glx was the most ^{15}N enriched at 17‰, with Asx, Pro, Ala all exhibiting similar values.

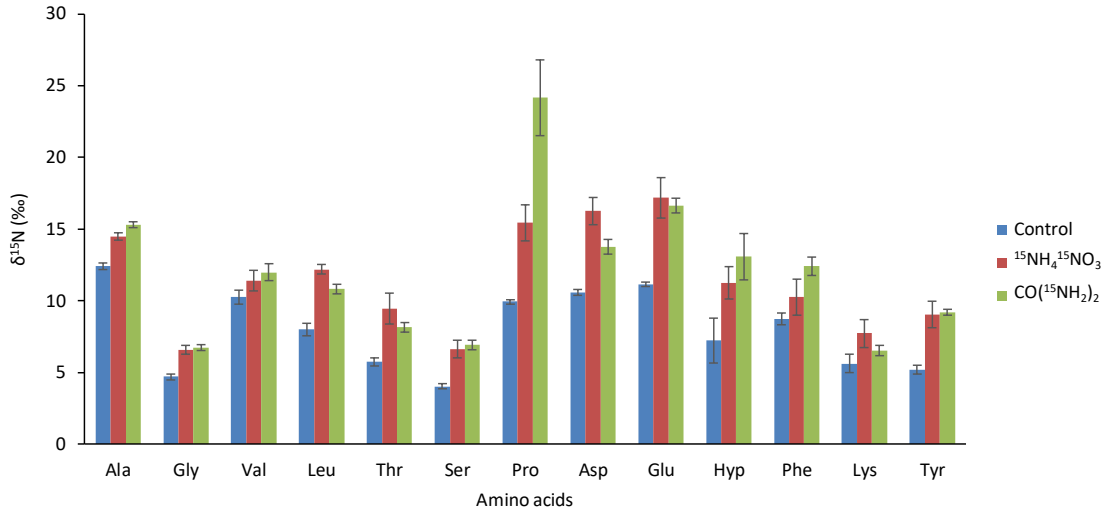


Figure 3.18. $\delta^{15}N$ values of the AA in the TC soil following growth of white clover (*Trifolium repens*), which was labelled through a split-root labelling technique injecting either $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ (30 mM at 98 atom%) or DDW for the control in the LC (mean ± standard error; n=5)

Unsurprisingly, from the applied ^{15}N-label in the LC, little incorporation was seen into the individual AAs in the TC soil (Table 3.18). For $^{15}NH_4{}^{15}NO_3$, incorporations ranged from 0.0003% for Hyp to a maximum of 0.0079% for Glx. Likewise, for $CO(^{15}NH_2)_2$ the lowest incorporation was seen for Hyp at 0.0004% and the maximum for Pro at 0.0121%, with a similar incorporation being seen for Glx at 0.0107%. This shows very low overall incorporation into the total hydrolysable AA pool or soil protein pool.

Table 3.18. Incorporation of the applied ^{15}N-label into individual AAs in the bulk TC soil (%) following the application of $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ (30 mM at 98 atom % to white clover (*Trifolium repens*) in the LC. Maximum values highlighted in bold.

	$^{15}NH_4{}^{15}NO_3$	$CO(^{15}NH_2)_2$
Alanine	0.0025	0.0040
Glycine	0.0027	0.0037
Valine	0.0010	0.0014
Leucine	0.0014	0.0014
Threonine	0.0020	0.0022
Serine	0.0016	0.0022
Proline	0.0038	**0.0121**
Aspartic acid	0.0034	0.0036
Glutamic acid	**0.0079**	0.0107
Hydroxyproline	0.0003	0.0004
Phenylalanine	0.0005	0.0011
Lysine	0.0037	0.0018
Tyrosine	0.0006	0.0007

3.4.6.4. Exudation of amino acids from clover in the labelling compartment following labelling with ^{15}N

Results in this section specifically address objective (vi), finding that clover produced a wide range of AA exudates (Figure 3.19), with the concentrations and relative amounts varying with the treatment applied (control or $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$). For the control and $^{15}NH_4{}^{15}NO_3$, Pro was shown to be exudated in the greatest concentrations, followed by Asx and Glx. However, for $CO(^{15}NH_2)_2$, Glx was exudated in the greatest concentrations, followed by Val and Asx. For the AAs present at the greatest concentrations in the control, substantially less was shown to be exudated by the plants labelled with $CO(^{15}NH_2)_2$). Of the total hydrolysable AA content produced by each plant, the control produced 0.28 ± 0.05 mg, $^{15}NH_4{}^{15}NO_3$

treatment 0.33 ± 0.05 mg and $CO(^{15}NH_2)_2$ 0.23 ± 0.04 mg. However, no significant difference was found between the total hydrolysable AA content of the exudates between treatments.

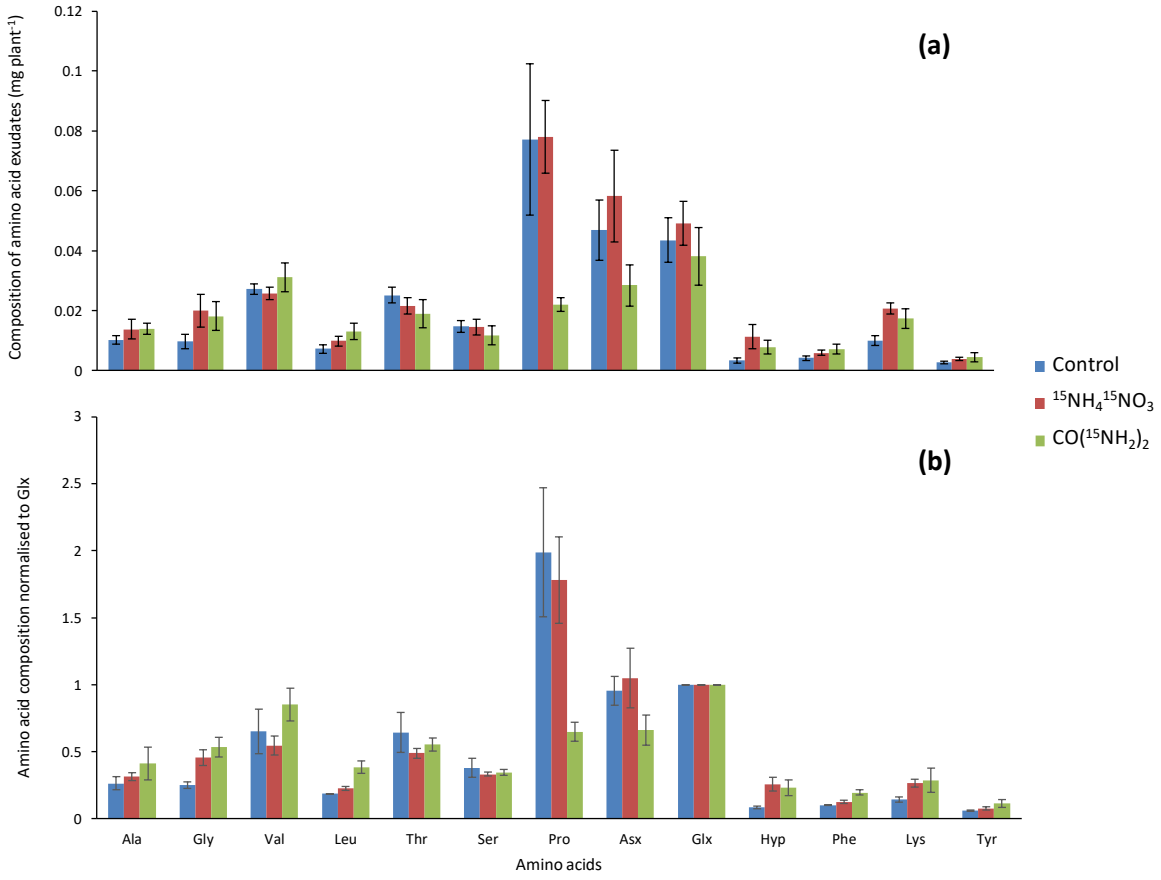


Figure 3.19. (a) Composition of AA exudates from white clover (*Trifolium repens*) [mg of AA produced by each plant in each incubation tube over the experimental period (mg plant^{-1})] in the LC following growth of clover and application of label through a split-root labelling technique injecting either $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ (30 mM at 98 atom %) or DDW for the control. Corrected for recovery rates of AAs from sand (Figure 3.13). (b) AA composition of clover exudates normalised to Glx (mean ± standard error; n=4).

3.4.6.5. Allocation of ^{15}N to amino acid exudates from clover following labelling with ^{15}N

Results in this section specifically address objective (viii), showing that AAs exudated from clover where plant roots were labelled with ^{15}N were highly enriched (Figure 3.20). $CO(^{15}NH_2)_2$ resulted in the AAs being more enriched with ^{15}N than with the application of $^{15}NH_4{}^{15}NO_3$, with the exception of Pro in the $^{15}NH_4{}^{15}NO_3$ treatment which was found to be ^{15}N enriched at 37 atom %. For $CO(^{15}NH_2)_2$, Leu was the most ^{15}N enriched AA at 46 atom %, while Hyp and Val showed the least ^{15}N enrichment in both treatments. The majority of the other AAs showed very similar ^{15}N enrichments.

For the control, Phe was shown to be slightly more ^{15}N enriched than Thr, while Leu, Lys and Gly were shown to be the least enriched in ^{15}N.

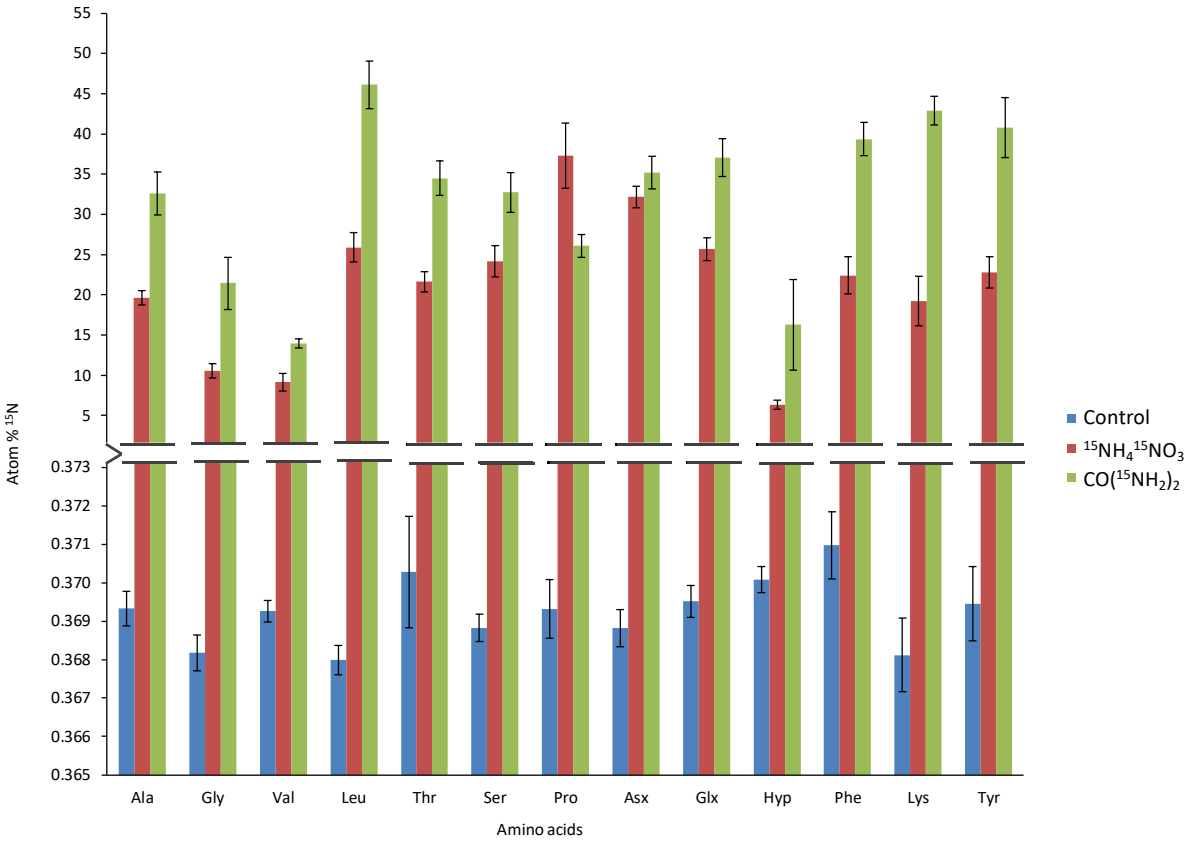


Figure 3.20. Atom % ^{15}N values of AA exudates from white clover (*Trifolium repens*) in the LC following growth of clover and application of label through a split-root labelling technique injecting either $^{15}NH_4{}^{15}NO_3$ or $CO(NH_2)_2$ (30 mM at 98 atom %) or DDW for the control (mean ± standard error; n=4). [Note scale in Atom % ^{15}N not $\delta^{15}N$ (‰)]

3.5. Discussion

3.5.1. Comparison of different ^{15}N-labelling techniques in rhizotrons

The initial experiment comparing different application methods provided a good starting point for differentiating between ^{15}N application methods and finding and adapting the most suitable method for use in future experiments. Fundamentally, this experiment yielded several points to be addressed in future experiments.

Firstly, results from this experiment were not as enriched as expected. This could firstly be due to a dilution effect on ^{15}N as N in this experiment came from three different sources: from labelling, soil uptake and N_2-fixation by the clover. Additionally, as a starting point, a relatively low concentration of ^{15}N (1mM $^{15}NH_4{}^{15}NO_3$ at 10 atom %) was used in comparison to other studies (see references in Table 3.1). This was most likely to have resulted in the low enrichment in the bulk $\delta^{15}N$ values seen. However, this was chosen as a reasonable starting point, as during previous enrichment studies by Cliquet et al. (1997) using 1 mM of NO_3^-, Asp and Ser yielded significant ^{15}N uptake when sampled 1 h after labelling. However, labelled compounds used by Cliquet et al. (1997) were at 99.9 atom % for NO_3^-, 92 atom % for Asp and 99.7% atom % for Ser. Whereas in this experiment $^{15}NH_4{}^{15}NO_3$ at 10 atom % was used, due to the fact that research has shown that at high levels of ^{15}N enrichment, ^{15}N discrimination and isotopic fractionation occurs, decreasing the accuracy of results (Mathieu et al., 2007; Carlisle et al., 2014; Tang and Maggi, 2012). A range of biological processes are seen to discriminate against ^{15}N, resulting in lower $\delta^{15}N$ values, such as nitrification and denitrification (Hobbie and Hogberg, 2012) and within plants, NO_3^- reduction (Carlisle et al., 2014), GS-GOGAT and transamination (Werner and Schmidt, 2002). In this experiment, the ^{15}N applied to each plant was more dilute at the start of the experiment than used by Cliquet et al. (1997) resulting in the lower than expected $\delta^{15}N$ values. Additionally, a concentration of 1 mM of $^{15}NH_4{}^{15}NO_3$ was chosen in this experiment as it is known that clover and ryegrass plants have a different response to N application, whereby at high N concentrations clover down regulates N_2-fixation (Ryle et al., 1976; Chapman et al., 1996; Griffth et al., 2000; Section 1.4). Furthermore, it has also been shown that the application of N fertiliser at low levels of 20 N kg/ha greatly reduces the number of nodules (Amba et al., 2013). Therefore, care needs to be taken over choosing a suitable concentration not to affect this balance. However, the level of labelling in this experiment was equivalent to 0.01 N kg/ha, therefore it is unlikely that we would reach levels which would have detrimental effects.

However, altering the $\delta^{15}N$ value of plant biomass is not straight forward. It is known that the overall $\delta^{15}N$ value of different plant parts is seen to be affected by a complex interaction of different processes, such as: the form of N taken up (e.g. NO_3^-), N transport within the plant, N transformations within the plant (NO_3^- reduction, NH_4^+ assimilation and metabolism of nitrogenous compounds), the $^{15}N/^{14}N$ fractionations during N assimilation as well as the losses from the plant (gaseous ammonium loss, leaf fall) (Robinson et al., 1998; Werner and Schmidt, 2002). This means it can be hard to predict the likely ^{15}N enrichment within a plant.

Where a low concentration of ^{15}N was used in the comparing ^{15}N-labelling methods experiments, calculations showed that a larger than expected percentage incorporation of the applied ^{15}N-label into the different plant parts (Table 3.4) and the soil (Table 3.6) occurred. This result was surprising due to the low $\delta^{15}N$ values, however, it provided a solid foundation for subsequent experiments, demonstrating that to increase the bulk $\delta^{15}N$ values (which will be fundamental to tracing N flow through two plant systems) a higher initial ^{15}N-labelling source was needed, either in terms of concentration of ^{15}N-labelled substrate or a higher atom %. Consequently, this was addressed in the subsequent experiment (verification of the leaf-labelling technique, Section 3.4.3). In future experiments $\delta^{15}N$ values should be two orders of magnitudes higher than in this experiment to ensure that there is sufficient exudation of ^{15}N-labelled compounds to the soil for detectable uptake into ryegrass systems, despite isotopic discrimination and losses.

Secondly, in adapting methods for future experiments, results from this experiment revealed the leaf-labelling technique to be promising for future experiments. This method has the advantage that it can be easily applied solely to clover plants, it is field applicable and when clover and ryegrass are allowed to co-exist in rhizotrons in future experiments, to calculate N-transfer between plants, the method could easily be applied without any complex set-up (which is a drawback associated with root-labelling techniques requiring splitting the roots between compartments). This approach has a reduced risk of contamination and enables ^{15}N enriched compounds to be applied easily to the clover plants without disturbing the root system. In experiments concerned with the application of a fertiliser treatment to leaves or roots (or a combination of the two), with time leaf-application has been shown to be the most beneficial, with plants treated in this way exhibiting higher levels of photosynthesis, transpiration, stomatal conductance rates, lamina ^{15}N incorporation and sucrose concentrations (Castle et al., 2007). In addition, the leaf-labelling technique is the most commonly used (Chalk et al., 2004)

as there have been concerns about the lack of field applicability of split-root labelling techniques (Wichern et al., 2008; Chalk et al., 2014). Furthermore, using the leaf-labelling technique, there is the possibility to further manipulate the plant system, for example, multiple pulses, removal of the ^{15}N source at different time points, or the application of a range of different compounds.

Thirdly, the leaf-labelling technique also offers promise for future experiments as it has been shown to result in a fairly uniform distribution of ^{15}N-label within different plant parts, which was not found using the root labelling technique, this finding is surprising but important. A number of the methods discussed in Table 3.1 have reported that the distribution of ^{15}N-label throughout the plant is not uniform. A uniform distribution of label is required for some calculations in order to calculate N-transfer from a legume to a non-legume (Chalk et al., 2014), calculating N-transfer is a fundamental to this work. However, it should be noted that Figure 3.4 also showed that the $\delta^{15}N$ values of different plant parts in the control were not the same, with $\delta^{15}N$ values consistently being higher in the roots. Furthermore, it has also been reported that temporal non-uniformity exists, whereby the enrichment of different plant parts declines with time due to plant growth, occurrence of N_2-fixation and the assimilation of soil N (Gardener et al., 2012). However, this experiment did not allow for a verification of temporal variation to be made, although the leaf treatment was studied in more detail in the subsequent experiment (Section 3.4.3). Furthermore, the time series in this experiment (Figure 3.3) produced by taking one leaf from each rhizotron, was not necessarily very accurate as there may not have been a uniform distribution of ^{15}N across every leaf and therefore it may not be representative of the labelling of the plant as a whole. Bulking plant parts together did not allow for an in-depth determination of the distribution of ^{15}N-label to be made. For example, it could not be determined whether there was uniform distribution throughout the whole root system, as it is often found that differences in ^{15}N enrichment exist between fine and course roots as well as nodules (Khan et al., 2002a, b; Rusell and Fillery, 1996; McNeill and Fillery, 2008). However, this method did provide an overview of plant uptake and enable some conclusions to be drawn, which could be built upon by sampling whole rhizotrons in later experiments.

Importantly, this initial experiment, enabled the ideal sampling point for each treatment to be determined. For the root labelling methods, continual uptake of the ^{15}N-label was shown (Figure 3.3), showing that as long as the plant system was enriched enough with ^{15}N to address the aims of the study, that the sampling point didn't matter. Conversely, the results from the

leaf-labelling technique showed that sampling at 100 h would be most suitable, thereby allowing experiments to be run within a single week. For this experiment, all ^{15}N-labelled treatments showed an initial decrease in $\delta^{15}N$ values following the introduction of the ^{15}N-labelled substrate (Figure 3.3). This could be due to the onset of N_2-fixation resulting in the dilution of ^{15}N in the plant. However, in other studies, where both legumes and non-legumes have been studied, similar decreases in $\delta^{15}N$ values have been observed (Kusliene et al., 2014). Therefore, this phenomenon may be due to plant processes discriminating against ^{15}N after a large initial application is introduced into the system, which has been shown to alter the isotopic fractionation of $^{14}N/^{15}N$ (Mathieu et al., 2007; Carlisle et al., 2014; Tang and Maggi, 2012). This finding is also important as it shows that sampling should not occur in the initial few hours after labelling.

3.5.2. Effect of different ^{15}N application methods on the distribution of root amino acids and ^{15}N allocation

To further study the effect of different ^{15}N-labelling techniques to plants, and to help establish the best method, $\delta^{15}N$ values of individual AAs were determined using the root only. This was done in order to establish whether the application method (shoots or roots) affected the partitioning of ^{15}N into individual AAs and would therefore have a subsequent effect on the partitioning of ^{15}N in compounds comprising root exudates, which could have important implications on N cycling for future experiments.

The results showed that there was no effect on the relative distribution of AAs or the partitioning of ^{15}N between them. In the clover roots, the major AA was shown to be Asx, which was unsurprising as Asn is the major transport molecule for N in clover and is found to be a major AA within clover roots (Paynel et al., 2001a). However, in the AA protocol used, the acid hydrolysis conditions results in Asn being completely hydrolysed to Asx (Fountoulakis and Lahm, 1998), hence the high concentration observed in Figure 3.6.

For the $\delta^{15}N$ values of individual AAs in the roots, Phe was shown to be enriched with ^{15}N relative to other AAs in all treatments. This phenomenon has been observed in a variety of plant species, for example; in wheat protein hydrolysates (Hofmann et al., 1995), in ryegrass (*Lolium perenne*) and rush (*Juncus effusus*) (but not moss *Brachythecium rutabulum*) (Bol et al., 2002), in dwarf and tall Red mangroves (*Rhizophora mangle* L.) (Smallwood et al., 2003)

and in cereals (Styring, 2012). Interestingly, the difference between the $\delta^{15}N$ values of Phe and the other AAs were greater in these studies. Phe plays an important role in plant growth, being a precursor for a wide range of secondary metabolites which have an important role in the overall structure of the plant. Together Phe and Tyr form the rigid polymer lignin, which is the second most abundant organic component in plant tissues (after cellulose) (Tzin and Galili, 2010; Nelson and Cox, 2013). Other secondary metabolites, including many phenolic compounds, such as flavonoids and stilbenes, provide a range of metabolic functions (Ferreyra et al., 2012; Stahlhut et al., 2015). The pathway which is responsible for the formation of these compounds, where Phe is a key intermediate, is the phenylpropanoid/phenylpropanoid-acetate pathway (Werner and Schmidt, 2002), with vascular plants having a very high turnover of Phe, as 30 to 45% of organic matter is derived from this pathway (Cantón et al., 2005). The enzyme involved in the first step of the pathway is phenylalanine-ammonia lyase (PAL) catalysing the reductive deamination of phenylalanine to cinnamate (Werner and Schmidt, 2002). Strying (2012) concluded that the reason behind the increased values seen is most likely to be because this enzyme is a branch-point enzyme with the kinetic isotope effect associated with this first deamination step in the progress which is likely to be expressed, leaving the residual phenylalanine enriched. Hermes et al. (1985) have previously found the kinetic isotope effect (k_{14}/k_{15}) associated with deamination on average to be 1.0106. It was also concluded that this enrichment shows the significance of the phenylpropanoid pathway in the production of organic matter (Cantón et al., 2005; Strying, 2012), therefore, it is likely that results in the comparing ^{15}N-labelling methods experiment are due to this pathway.

Despite the high quantities of Asx found in the roots and the high $\delta^{15}N$ values of Phe, the percentage incorporation of the applied ^{15}N-label showed no significant difference between the treatments, with the greatest incorporation being observed for Asx, Hyp and Lys depending on the treatment applied, Thr was also observed to be an important pool for the ^{15}N-label. Lys is biosynthesised from Asp (as well as Thr, Asn, Met) (Nelson and Cox, 2013). Therefore, it is unsurprising that high levels of incorporation of the ^{15}N-label were seen, due to the high amount of Asx seen in the roots which could be then biosynthesised into new AAs. Furthermore, Lys has several functions within plants, including contributing to mitochondrial metabolism and ATP production (Araújo et al., 2010; Hildebrandt et al., 2015). However, the biosynthesis of Hyp is unrelated to the other AAs that were shown to incorporate ^{15}N-label in high amounts, it being a post-translation metabolite of Pro through hydroxylation (Wu et al., 2011) and acting as a component of plant cell walls (Lamport and Northcote, 1960; Cassab, 1998). This would

agree with the considerable amount of plant biomass produced in this experiment, emphasising the active growth and biosynthesis to create new plant walls.

3.5.3. Uptake and distribution of ^{15}N over-time *via* the leaf-labelling technique in rhizotrons

This experiment built on findings from the comparing ^{15}N-labelling methods experiment (Figures 3.3-3.7, discussion 3.5.1) which suggested that the leaf-labelling technique may be a promising method to apply ^{15}N compounds to plants. The aim was to establish the most appropriate sampling time and to increase the amount of ^{15}N uptake over-time, in order to develop a robust method for studying the N-transfer between two plants. Therefore, the labelling solution was increased to 30 mM from 1 mM (at 10 atom %). This experiment also enabled the fate of ^{15}N into all plant parts to be studied over-time.

Even with the concentration increased by 30 times, only low $\delta^{15}N$ values were observed. A low percentage incorporation of the applied ^{15}N-label into plant parts was observed, with the verification of the leaf-labelling technique experiment showing similar values to the comparing ^{15}N-labelling methods experiment, despite the increased concentration (Table 3.4 and 3.6 versus Figure 3.8 and 3.9). These results suggested that clover was ineffective at taking up $^{15}NH_4{}^{15}NO_3$ through its leaves, however this has been shown to be an effective method of ^{15}N enriching plants in other studies (see references in Table 3.1). This finding may be due to clover preferring to take up N through its roots following the natural pathway of N assimilation, decreasing the amount of uptake needed and required by the plant through its leaves. Furthermore, lower than expected $\delta^{15}N$ values could have been a result of active N_2-fixation, as nodules were present on roots collected from the experiment. It has been found that nodulation of legume roots lowers the overall ^{15}N enrichment of the root, due to dilution by the N_2-fixed from the air (Jensen, 1996b), which can then be further distributed around the rest of the plant. However, there are many possible reasons for the low uptake of the label seen. Studies have also shown that the uptake efficiency of the ^{15}N-label depends on the climatic conditions and the plant growth stage, as the uptake of solution is determined by the transpiration stream and plant N accumulation (Hogh-Jensen and Schjoerring, 2001), therefore, conditions may not have been favourable for uptake resulting in low $\delta^{15}N$ values.

More variation is seen in Figure 3.8 than in the comparing ^{15}N-labelling methods experiment. In the verification of the leaf-labelling technique experiment, clone clover plants were used, so the variation seen must have arisen from environmental factors. Khan et al. (2002a) also observed that when using different $CO(^{15}NH_2)_2$ concentrations variation increased as higher concentrations were used. However, high concentrations of $CO(^{15}NH_2)_2$ are known to result in leaf damage (tissue necrosis) (Bremner, 1995; Gooding and Davies, 1992; Palta et al., 1991) and Khan et al. concluded that this was the most likely reason for the variation. However, in this study $^{15}NH_4{}^{15}NO_3$ which is not known to have such effects on leaves was used. Another possible cause could be the influx of ^{15}N into the plant system, which could have altered the $^{14}N/^{15}N$ isotopic fractionation factor, which could have decreased the overall enrichment (Mathieu et al., 2007. However, effects should have been minimised as for the initial experiments, 10 atom % was chosen as a starting point as research has shown that at high levels of ^{15}N enrichment, changes occur in ^{15}N discrimination and isotopic fractionation occurs, decreasing the accuracy of results (Mathieu et al., 2007; Carlisle et al., 2014; Tang and Maggi, 2012).

For the AAs, the high concentration of Asx in the stolon (Figure 3.9a) confirms the transport role of the stolon as well as the fact that active transfer of N is taking place with the application of $^{15}NH_4{}^{15}NO_3$ to the leaves and its subsequent distribution throughout the plant. The role of the stolon for nutrient transport and Asn as the major transport molecule for N, are further supported and expressed by the percentage incorporation the ^{15}N-label (Figure 3.12a), with incorporation into Asx being the greatest.

Observations from this experiment also show how the distribution of AAs in the roots varies between the two experiments. The high concentration of Asx in the roots is more apparent in the comparing ^{15}N-labelling methods experiment (Figure 3.6) than in the verification of the leaf-labelling technique experiment (Figure 3.10c) with very similar concentrations of all AAs. This would suggest differences in the nutrient status of the plants, where the plant roots had become fairly compacted in the comparing ^{15}N-labelling methods experiment, with substantial biomass being produced (Table 3.5 compared to Table 3.9). A greater amount of biomass would require more nutrient uptake from the soil with N being transported by Asn. This could suggest, that the leaf-labelling technique in the verification of this method experiment provided all the N requirements to the plant, despite the vials of substrate being in place for 72 h only. However, at 0 h where no ^{15}N-label was supplied and the plant did not have any additional

supplemented N source, the concentration of Asx was observed to be low, especially in comparison to other AAs, suggesting no N transport in the roots. Therefore, it is unclear why the relative concentration of Asx varies between experiments, although it is likely to be related to the plant biomass produced and different initial growth stages in the experiments.

For the AAs in the leaves, Glx was shown to be at slightly greater concentrations than Asx, however, the concentrations were not substantially greater than the other AAs (Figure 3.10b). Additionally, similar percentage incorporation into individual AAs were observed (Figure 3.11b), showing that the leaves were synthesising new plant biomass, especially when comparing with results from the stolon.

In the comparing ^{15}N-labelling experiment, Phe was the most ^{15}N enriched AA observed in the roots (Figure 3.7) which also corresponded to previous studies on plants (Section 3.5.2). However, for this experiment the pattern was not as distinct (Figure 3.11a, b, c). AAs in the stolon, leaves and roots, the δ^{15}N values of Tyr are often seen to be higher or comparable to Phe. Both Phe and Tyr have been found to have similar roles within plants, as precursors for the rigid polymer lignin (Tzin and Galili, 2010; Nelson and Cox, 2013), and both are key substrates in phenylpropanoid biosynthesis (Werner and Schmidt). Therefore, similar mechanisms which resulted in the high δ^{15}N value of Phe described previously (Section 3.5.2) are likely to have resulted in high δ^{15}N values for Tyr. Furthermore, catabolism of Phe occurs through an initial conversion to Tyr where no N bonds are broken (Nelson and Cox, 2013), therefore, no isotopic discrimination occurs, resulting in similar values for these two AAs. However, this is thought not to occur within plants, as no Phe hydroxylase homologue has been found (Hildebrandt et al., 2015).

For the soil, little to no change was seen in the δ^{15}N values of both the bulk soil (Figure 3.8) and the AAs (Figure 3.11d). Although interestingly, the bulk soil showed the largest incorporation of the applied ^{15}N-label compared to the plant parts studied (Figure 3.9), this is probably due to the calculation taking in account the mass of material the ^{15}N-label was incorporated into, for example 100 g of soil compared to ~0.4 g of leaf material. Critically, these results showed that significantly more enrichment of the soil is needed if N-transfer is to be calculated between plants. This means that the δ^{15}N values of the plants need to be further elevated, since when using the leaf-labelling technique, the only way that bulk δ^{15}N values of the soil can become enriched is through plant rhizodeposition, such as exudates.

In terms of developing a method for future experiments, findings from this experiment along with the comparing ^{15}N-labelling methods experiment, allowed a sampling time of 100 h to be established, as bulk $\delta^{15}N$ values peaked at 100 h for both studies, although varying results were seen for the AA $\delta^{15}N$ values. Despite this, choosing a sampling time of 100 h for future experiments allows the experimental labelling period to be conducted within a single week which will assist in the conduction of experiments. Furthermore and crucially, the experiment showed that either a higher concentration of ^{15}N or a higher atom % is required. However, previous experiments introducing a ^{15}N-label to plants often report a maximum concentration of 30 mM (see references in Table 3.1), therefore, the atom % should be increased in future experiments.

3.5.4. Potential for ^{15}N uptake via the leaf-labelling study by eliminating background nitrogen

Previous investigations undertaken in this chapter, using the leaf-labeling technique, have shown a very low potential for uptake of ^{15}N at both 10 mM and 30 mM $^{15}NH_4{}^{15}NO_3$ (at 10 atom %), therefore, uptake of additional N from the soil was eliminated as this could have been a possible cause of the low uptake observed. Further to this, the atom % of ^{15}N in the labelling substrate was increased to 98%. Whilst using a low atom % should minimise the amount of isotopic discrimination through biological processes, it also resulted in low ^{15}N uptake making it impossible to study N-transfer between plants in later chapters. As a result of this change, much greater levels of ^{15}N enrichment were seen in all plant parts (Figure 3.13). However, interestingly the experiment also resulted in very low incorporation rates of the applied ^{15}N-label into bulk plant $\delta^{15}N$ values (Table 3.12), especially considering the increased atom %. Although, this is in agreement with the two previous experiments, suggesting that clover leaves do not assimilate the labelling solution efficiently through th leaves.

Additionally, in this experiment using a different ^{15}N substrate was investigated as another method for increasing the ^{15}N enrichment of the plant. Results showed greater ^{15}N enrichment was achieved using $CO(^{15}NH_2)_2$ than $^{15}NH_4{}^{15}NO_3$, resulting in approximately double the enrichment (Figure 3.13). $^{15}NH_4{}^{15}NO_3$ was chosen as the initial ^{15}N-labelling source, since it is the most commonly applied fertiliser in Great Britain (Defra, 2017). Furthermore, $^{15}NH_4{}^{15}NO_3$ was also chosen to decrease the potential for a non-uniform distribution of label. Previous studies have shown that the use of dual labelled NO_3^- and NH_4^+ minimises this affect as well

as enabling labelling of the whole plant (Murray and Clements, 1998). Other studies have shown that intra-plant variation tends to occur when NO_3^- is the sole source of N, while when NH_4^+ is the sole source of N little variation tends to occur. This is due to N assimilation occurring through two different pathways depending on N source. For NH_4^+ assimilation occurs at the root to avoid toxic accumulation through the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway (Section 1.3.1), this results in the organic N present in roots and shoots being a result of one assimilation process. However, NO_3^- assimilation occurs through the nitrate-reductase-nitrite reductase pathway which can take place in the roots or the shoots. It is found that enrichment of the $\delta^{15}N$ values of NO_3^- occurs due to fractionation during the assimilation processes. Furthermore, this results in the $\delta^{15}N$ values of leaves tending to be greater than roots, as the NO_3^- available for assimilation in the leaves has already been exposed to assimilation in the roots (Yoneyama and Kaneko, 1989; Evans et al., 1996; Evans, 2001). Additionally, different plant ^{15}N enrichments can also be found where NH_4NO_3 is applied either as $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$. Inselsbacher et al. (2013) showed that incorporation into plant N is slightly lower for $^{15}NH_4NO_3$ than $NH_4{}^{15}NO_3$ over-time. Furthermore, some differences in $\delta^{15}N$ values cannot be avoided (as seen in the control) as many biological processes discriminate against ^{15}N and reallocation of N during growth can cause differences between plant parts (Evans, 2001). These previous studies support the choice of dual-labelled $^{15}NH_4{}^{15}NO_3$.

Further to the initial selection of $^{15}NH_4{}^{15}NO_3$ in experiments was due to the conflicting evidence of different fertiliser applications on nodulation and N_2-fixation. It is well known that the application of fertilisers affects the processes of nodulation and N_2-fixation, with these processes being energetically more costly than N substrate uptake (Phillips, 1980; Ryle et al., 1984; Saito et al., 2014). However, increased inhibitory effects have not only been seen with N concentration but also with the substrate applied (Svenning et al., 1996; Bollman and Vessey, 2006; Barbulova et al., 2007). For pea (*Pitsum Sativum*), white clover (*Trifolium repens*) and soybean (*Glycine max*) NO_3^- is found to have a more inhibitory effect on N_2-fixation, nodulation, nodule mass than NH_4^+ (Imsande, 1986; Svenning et al., 1996; Bollman and Vessey, 2006) However, Guo et al. (1992) found the inhibitory effect to be greatest with NH_4^+ in faba bean (*Vicia faba*) and white lupin (*Lupinus albus*). However, the interaction between plant processes and fertiliser application is complex, as NH_4^+ is found in some cases to stimulate nodulation, but when applied as a combination of NO_3^- and NH_4^+, total dry mass and

total N are increased (Waterer et al., 1992; Gulden and Vessey, 1997; Bollman and Vessey, 2006). Therefore, making the choice of which fertiliser is the best to apply is a complex process.

$CO(^{15}NH_2)_2$ was chosen as an alternative ^{15}N enrichment substrate to determine if higher $\delta^{15}N$ values could be achieved. $CO(NH_2)_2$ is the most commonly used fertiliser in agriculture worldwide (Gilbert et al., 2006). $CO(^{15}NH_2)_2$ is an important N metabolite (Mérigout et al., 2008), and within legume grazed pastures this represents a large input from excreta (Whiteahead, 1970; Ledgard, 2001). The use of $CO(NH_2)_2$ has increased more than 100-fold over the past four decades, now contributing to over 50% of global fertiliser usage, with this increase likely to continue (Gilbert et al., 2006). $CO(NH_2)_2$ application has increased substantially because of its low manufacturing costs, high N content (46%; greater than that of NO_3^- and NH_4^+), the fact that it can be applied in various forms (solid or liquid) and it is also more stable, and less explosive than alternatives making it easier to transport. (Gilbert et al., 2006; Arkoun et al., 2012). $CO(NH_2)_2$ makes a good ^{15}N substrate for ^{15}N-labelling studies due to it being mobile for plant uptake and non-polar (Fusetc et al., 2010). $CO(^{15}NH_2)_2$ introduced *via* leaf labelling has been successfully used in studies and has resulted in ^{15}N labelling of all plant parts (Hogh-Jensen and Schjoerring, 2001). However, for field experiments $CO(^{15}NH_2)_2$ has seldom been used (McNeill et al., 1997, Hogh-Jensen, Schjorring, 2001; Arkoun et al., 2012; Harty et al., 2017).

When $CO(NH_2)_2$ is applied to soils, it is immediately hydrolysed by an enzyme produced by most soil microorganisms and plant species, urease, to NH_4^+, (Watson et al., 1994; Gill et al., 1999) with subsequent nitrification to NO_3^-. After conversion, the efficiency of $CO(NH_2)$ for plant uptake is seen to decrease greatly due to losses as gaseous NH_3; or through NO_3^- which has high mobility due to not being absorbed onto soil particles and is subject to leaching losses (Terman, 1980). Therefore, $CO(NH_2)_2$, NH_4^+ and NO_3^- often exist simultaneously within the rhizosphere for plant uptake (Mérigout et al., 2008). Urease inhibitors are frequently applied which slow the rate $CO(NH_2)_2$ is hydrolysed and converted to NH_4^+, reducing the problems which can be associated with $CO(NH_2)_2$-based fertilisers, as well as increasing their efficiency and allowing plant uptake (Trenkel, 2010; Gill et al., 1999). However, plants can uptake urea *via* their roots or leaves prior to its hydrolysis to NH_4^+ and CO_2 (Hine and Spent, 1988; Krogmeier et al., 1989; Gerendás et al.,1998; Mérigout et al., 2008; Trépanier et al., 2009). The co-existence of $CO(NH_2)_2$, NH_4^+ and NO_3^- for plant uptake in this study, is likely to be one of the reasons behind the higher ^{15}N enrichment compared to applying $^{15}NH_4{}^{15}NO_3$ alone.

In comparing studies using different fertilisers ($CO(NH_2)_2$, NH_4^+ and NO_3^-), a range of different effects have been observed. The effectiveness of different fertilisers is often determined by ion exchange principles (Arkoun et al., 2012). A range of different plant species have shown preferential uptake of $CO(NH_2)_2$ compared to other N forms. Orchid plants (*Phalaenopsis* cultivars) were shown to absorb from solution 47% of the total amount of $CO(NH_2)_2$, 41% NH_4^+ and 12% NO_3^-, with further analysis showing no hydrolysis of $CO(^{15}NH_2)_2$ before root absorption (Trépanier et al., 2009). For ryegrass and white clover pastures (*Lolium perenne* and *Trifolium repens*) plant uptake and recovery of the applied fertilisers was seen to be greater with $CO(NH_2)_2$ than $(NH_4)_2SO_4$ or KNO_3 (Keeney and Maccregor, 1978). Similarly, Imsande (1988) noted some beneficial effects of growing soybean on $CO(NH_2)_2$ alone compared to NO_3^- in well-nodulated plants, with increased transpiration rates, larger seeds and increased total plant mass. Imsande and Schmidt (1998) also found that well-nodulated soybeans assimilated the most N when $CO(NH_2)_2$ was applied (compared with NO_3^-) and increased with concentration used, similar effects were observed on yields of non-nodulated and nodulated soybeans, as well as seed N concentrations. $CO(NH_2)_2$ has also been shown to have less or even no inhibitory effect on nodule development and N_2-fixation compared with other fertilisers (Vigue et al., 1977; Imsande, 1988; Guo et al., 1992; Cheema and Ahmad, 2000). Similarly, Paradiso et al. (2015) showed that nodulation increased with the application of $CO(NH_2)_2$ compared to NO_3^-, although $CO(NH_2)_2$ was seen to limit overall growth and seed yield especially in the early developmental stages of plant growth. Other studies have shown that of the reduced sources of N available ($CO(NH_2)_2$, amides, and NH_4^+ salts of citrate, malate, fumarate, succinate, and sulfate) that $CO(NH_2)_2$ caused the least damage to plants. However, overall soybean seedlings grow better with NO_3^- than $CO(NH_2)_2$ (Lahav et al., 1976). Watson (1987) showed that in perennial ryegrass (*Lolium perenne*) recovery from the shoots, roots and soil was lower with $CO(NH_2)_2$ than NH_4NO_3 (60% verus 95%, respectively). $CO(NH_2)_2$ has also been shown to be the most efficient fertiliser with the lowest N losses (especially when urease inhibitors are used) However, the amount of ^{15}N taken-up by plant tissues was seen to be higher with NH_4NO_3 in oilseed rape (*Brassica Napus* L.) (Arkoun et al., 2012). Some studies have even reported reduced plant growth with $CO(NH_2)_2$ fertilisation, for example, in pine (*Pinus taeda* L.), although this made plants less susceptible to the effects of drought (Faustino et al., 2015). In addition, it resulted in less biomass produced by white clover (*Trifolium repens*) with $CO(NH_2)_2$ application than NO_3^- (Castle et al., 2007). The varying results seen in these studies on the application of different fertilisers, makes it critical to determine the most suitable ^{15}N carrier for the specific plant species studied, with results in this chapter revealing a

preference in uptake of $CO(^{15}NH_2)_2$ rather than $^{15}NH_4{}^{15}NO_3$ in white clover in terms of ^{15}N enrichment of plant parts, with no effects on plant growth being observed. The N content in the plant was increased when either substrate was applied. Due to time constraints no other parameters which determine the effects of the substrate on the plant (e.g. nodulation) were assessed.

In terms of developing a method for future experiments, the findings from this experiment eliminating background N suggested that $CO(^{15}NH_2)_2$ may provide a better ^{15}N-labelling source for clover than $^{15}NH_4{}^{15}NO_3$, resulting in greater $\delta^{15}N$ value enrichment of plant parts. Results also show that submerging clover leaves in the ^{15}N-label solution has a low potential for uptake, questioning the use of this technique in clover. However, this technique has been successfully used in subterranean clover (*Trifolium subterraneum*), showing similar ^{15}N enrichment values with plants growing in river gravel and labelled with $^{15}NO_3^-$ or $^{15}NH_4^+$ to those obtained with $CO(^{15}NH_2)_2$, the experiment conducted in this chapter, however three clover leaves were submerged compared to two. The method was then later successfully used to measure N-transfer between plants in soil, submerging four leaves from each clover plant in $(^{15}NH_4)_2SO_4$, although ^{15}N enrichments were lower, results did allow transfer to be calculated (Ledgard et al., 1985). Therefore, maybe the method needs more adjustments to substantially enrich white clover. For example, Khan et al. (2002a) showed that by making small adjustments to get more ^{15}N enrichment into the roots, the location of the vial containing the ^{15}N-label needs altering, finding that in chickpea (*Cicer arietinum*) the roots became more enriched with ^{15}N when the leaves were labelled at the base of the stem. Therefore, further investigation and adjustments could be made to the leaf-labelling technique, however, it is unlikely that this will vastly increase the incorporation of the ^{15}N-label, which lead to investigation of the split-root labelling technique following the natural pathway of assimilation (Section 3.4.6).

3.5.5. Recovery of amino acids from sand

Recovery of AAs from sand was initially investigated due to problems obtaining results on AA exudation from clover growing within sand in the eliminating background N experiment. The recovery experiment showed that while it is possible to recover AAs from sand, AAs are recovered at different rates, which has important implications when quantifying exudates.

Lys was found to be retained by the sand in the greatest amounts (over 70% loss) most likely due to the positively charged Lys being retained by the negative charges of silicate minerals. Arg and His are also basic AAs, however, these were not present in the mixed AA standard to test this theory. The reasons behind this is that to date no reliable method exists to reliably quantify Arg due to it only partly derivatising to it's NAIP esters and decomposition on the GC column, resulting in poor chromatography which is required for quantification (Kendall, 2017). While His is destroyed during acid hydrolysis (Block, 1940). However, the chemical properties of the AAs doesn't fully explain the losses seen, which are shown in Table 3.19. This shows that whilst Asp and Glu are acidic in nature they are also lost in relatively high concentrations.

Other studies have investigated the adsorption of AAs onto sea sand, apatite, illite, montmorillonite and hematite, and have found that AAs with charged R groups are adsorbed onto these surfaces more than AAs with uncharged R groups (Lahav and Chang, 1976; Ben-Taleb et al., 1994; Zaia et al., 2002). This explains why the AAs with a charged side chain such as Lys or Glx are adsorbed more on sand than AAs without a charged side chain such as Gly; although this cannot fully explain the findings as their charges are seen to differ, and cannot explain the loss of Val, Phe or Tyr. It is also likely that this problem could be resolved by rinsing the sand with HCl, however, if the sand still contains roots this could potentially hydrolyse a proportion of root AAs which would interfere with results. Despite this, the findings show the importance in quantifying the recovery of AAs, especially when collecting such small concentrations of samples, such as plant exudates.

Table 3.19. Properties of AAs studied in this project (in elution order).

Amino acid	Chemical properties	Physical properties
Alanine	Aliphatic	Nonpolar
Glycine	Aliphatic	Nonpolar
Valine	Aliphatic	Nonpolar
Leucine	Aliphatic	Nonpolar
Threonine	Non-aromatic hydroxyl	Polar (uncharged)
Serine	Non-aromatic hydroxyl	Polar (uncharged)
Proline	Cyclic	Nonpolar
Aspartate	Acidic	Polar (negatively charged)
Glutamate	Acidic	Polar (negatively charged)
Phenylalanine	Aromatic	Nonpolar
Lysine	Basic	Polar (positively charged)
Tyrosine	Aromatic	Nonpolar

3.5.6. Determining the suitability of the split-root labelling technique

3.5.6.1. Plant enrichment with ^{15}N

Following the natural pathway of N assimilation, the use of a split-root labelling technique adapts the method of applying ^{15}N-labelled fertiliser to the soil allowing the distinction between soil and root-borne nitrogenous compounds (Merbach et al., 2000). The split-root labelling technique is the only technique which allows continuous labelling of all plant parts with ^{15}N (Sawatsky and Soper, 1991; Jensen, 1996a). It was initially ruled out due to its more complex application (cf. the leaf-labelling technique), requiring the roots to be sufficiently developed before transplanting plants and separating the root visually into two equal parts. Half of the roots were placed into an artificial medium where additions of ^{15}N could be applied, with the other half being placed within soil and allowing roots to intermingling with the companion species. This method can result in extensive disturbance of the roots, especially when being fed through glass tubes. Therefore, this method would not be suitable for a wide range of plants, especially those with dominant tap roots. This method has been noted for its lack of field applicability (Wichern et al., 2008; Chalk et al., 2014), due to mostly being carried out within a greenhouse setting, although it has seen limited use in the field (Martin et al., 1991).

Very few studies exist which compare the use of ^{15}N shoot labelling aboveground to the use of root belowground methods (Jensen, 1996b; Mahieu et al., 2007). Merbach et al. (2000) noted that it was difficult to achieve sufficient labelling of plants using shoot labelling. The use of a

split-root labelling technique offers some advantage over the leaf-labelling technique, as it allows high enrichment to be achieved through continuous application of a ^{15}N enriched substrate *via* a natural pathway of N assimilation. Typically, this has been reported to promote incorporation in all N pools of the plant and should also uniformly ^{15}N-label all the compounds available for N transfer (Jensen, 1996a). This technique also permits earlier absorption of ^{15}N by the plant compared to the leaf-labelling technique (as shown in the comparing ^{15}N-labelling methods experiment, Figure 3.3). This method also allows the concern of $CO(NH_2)_2$ application to the leaves causing leaf damage to be overcome (Bremner, 1995; Gooding and Davies, 1992; Palta et al., 1991), as well as, preferential labelling of the leaves. There is also a danger of run-off of the highly enriched labelling solution into the soil when using a leaf-labelling technique (Khan et al., 2002a). This shows, that all methods have their drawbacks, ultimately the choice of method depends on the study aims.

Typically, the use of a split-root labelling technique seems to be widely used in studying rhizodeposition (Schmidtke, 2005; Mahieu et al., 2007; Wichern et al., 2008; Fustec et al., 2010; Verman et al., 2018). Although most studies using it to study N-transfer are over 15 years old, with only one recent study (van Kessle et al., 1985; Martin et al., 1991, Frey and Schüepp et al., 1993; Ikram et al., 1994; Jensen, 1996; Johansen and Jensen, 1996; Mårtensson et al., 1998; Parnamawati and Schmidtke, 2003; Génard et al., 2016), demonstrating the preference for leaf-labelling techniques.

Despite a much higher ^{15}N enrichment of all plant parts being achieved with the split-root labelling technique compared to the leaf-labelling technique, one of the major disadvantages that was seen in the results was the non-uniform distribution of the applied ^{15}N-label, despite the suggestion that following the natural pathway of N assimilation should promote incorporation into all N pools (Jensen, 1996a). This finding has also been observed in other studies with the plant roots to which the ^{15}N-label is applied retaining the majority of the ^{15}N-label (Schmidtke, 2005; Mahieu et al., 2007). Reining et al., (1995) also found that considerable quantities of ^{15}N were taken up into spring wheat (*Triticum aestivum*) using a split-root technique, with 90% of the label being recovered in the shoot, 3% in the roots (TC) and 7% was released into the soil. However, the percentage of ^{15}N-label remaining in the LC roots was not measured, the results from this study also showed how the ^{15}N enrichment decreases away from the labelling source. The ^{15}N-label is often found to be a significant proportion of the total plant N, for example, Merbach et al. (2000), using a split-root technique, found this to be

around 30% thereby finding an influence on the rhizodeposition patterns which were also affected by the amount of nutrients added and the level of enrichment of the ^{15}N tracer. This shows that while the findings in this chapter were not uncommon, the split-root labelling technique allowed sufficient plant ^{15}N enrichment to be achieved and consideration needs to be taken when choosing appropriate calculations for N-transfer (Chapter 4) due to the non-uniform distribution of the applied ^{15}N-label.

3.5.6.2. Bulk soil and amino acid enrichment with ^{15}N

As well as enabling significantly more enrichment in different plant parts than the leaf-labelling technique, the split-root technique also resulted in ^{15}N enrichment of the soil. The use of $CO(^{15}NH_2)_2$ resulted in greater ^{15}N enrichment of the soil compared to $^{15}NH_4{}^{15}NO_3$ or the unlabelled control. However, this only represented minimal transfer from the roots into the soil, of 0.58% for $CO(^{15}NH_2)_2$ and 0.37% for $^{15}NH_4{}^{15}NO_3$.

Mahieu et al. (2007) found that the split-root labelling technique was less efficient at labelling plant organs and resulted in less transfer of ^{15}N to the soil than shoot labelling *via* a cotton-wick for pea (*Pisum Sativum*). However, the ratio of Ndfr: BGN (ratio of the proportion of total N derived from rhizodeposition to total below ground part N) was found to be higher with the split-root technique than with shoot labelling (65% *vs* 45%, respectively), the authors concluded that these results suggest an over-estimation using the split-root technique. Merbach et al. (2000) also concluded that the split-root labelling technique leads to overestimation of net rhizodeposition compared to the use of $^{15}NH_3$ fumigation and pre-cultivation of plants in ^{15}N growth medium. Therefore, it is likely that the higher soil ^{15}N enrichment seen in this study could have been as a result of the labelling method. Furthermore, this means that it can be hard to compare plant ^{15}N transfer to the soil using a root and leaf-labelling technique, meaning that different labelling techniques are likely to affect plant-to-plant N-transfer, having important implications on the findings in Chapter 4.

3.5.6.3. Exudation from clover

An important factor in understanding N-transfer between plants, is determining the different forms in which N can be released by plants and transferred between plants, or rhizodeposition. Two main pathways for rhizodeposition of N have been identified as: (i) senescence, decomposition and decay of roots and nodules, and (ii) living plant exudation of soluble N compounds (Fustec et al., 2010), which in turn play an important role in nutrient cycling of C and N (Lesuffleur at el., 2007). The amounts of N in rhizodeposits is found to range between 4 and 71% of the total N assimilated by the plant. For legumes, the average was 16% and furthermore, of the total belowground plant biomass an average of 73% was found for all studies reviewed by Wichern et al. (2008). Fustec et al. (2010) also reported the same amount of variation between studies looking at rhizodeposition, showing that greater understanding is still needed to increase our understanding of N cycling in soils.

Studying rhizodeposition, such as plant exudates, is known to be inheritably difficult. This study concentrated solely on the AA plant exudates, due to the fact that organic N concentrations in soil are far greater than inorganic N, with AAs making up a large fraction of this (Schulten and Schnitzer, 1998; Friedel and Scheller, 2002; Jones and Kielland, 2012). Friedel and Scheller (2002) found that of the total soil N pool, hydrolysable AAs comprised about 20-50%, making them an important N source for understanding N-transfer between plants. Despite this, studies have shown that the water-soluble exudates produced by plants mainly comprise carbohydrates and organic acids, with only a small fraction being AAs (Merbach et al., 1999; Hütsch et al., 2002). Furthermore, studies have shown that N containing compounds released by plants include, NO_3^-, NH_4^+ and AAs (Rovira, 1956; Brophy and Heichel, 1989; Wacquant et al., 1989) and plants are found to naturally cycle AAs and exude them across the root cell plasma membranes; a process driven by differences in concentration (Phillips et al., 2004). For legumes, alfalfa has been found to release significantly more NH_4^+ than soybean, for both plant species. However, passive leakage of amino-N was not a primary mechanism for N release from their roots (Brophy and Heichel, 1989). Similarly, studies on white clover have found that larger quantities of NH_4^+ are released from plants than AAs (Paynel et al., 2001a; Paynel and Cliquet, 2003). Studies commonly show that NH_4^+ is the main compound released by plants, due to it being the main product of the nitrogenase enzyme (Fustec et al., 2010). This phenomenon is not found in all plants. When looking at the chemical characterisation of ^{15}N material released into the soil by spring wheat (*Triticum aestivum*), Reining et al. (1995) found similar concentrations of NH_4^+ and AAs. Furthermore, the significance of plant exudation is still unknown, with studies showing that exudates have a

limited role in rhizosphere nutrient mobilization (Jones et al., 1994), although it has been found that exudates play a role in determining the size of the rhizosphere microbial population (Jones and Darrah, 1994). However, these studies further show that collecting and quantifying the AA exudates and understanding their importance in bulk soil for the N-transfer between plants is even more complex.

An initial attempt to quantify AA exudation from plants was made with the rhizotrons in the eliminating background N experiment, growing plants solely in sand (Section 3.4.4). However, due to the low volume of plant biomass produced especially in relation to the amount of sand provided to support root growth, quantification was not possible. This might have also been a result of plant reuptake of exudates resulting from the nutrient-poor conditions of the sand, as plant exudation comprises a net release of compounds from both the influx and efflux (Jones and Darrah, 1994). Adaptation of the split-root labelling technique allowed plants to be substantially enriched with ^{15}N (Figure 3.16), supporting adequate plant growth with half of the plant roots growing within soil, but allowed simultaneous collection of plant exudates. Therefore, the split-root labelling technique was multi-functional in addressing different aims of the study. However, a major disadvantage of this technique is that it only considers part of the root system, so it is likely to underestimate the true value of rhizodeposition (Schmidtke, 2005; Rroço and Mengel, 2000; Merbach et al., 2000), although, it is important to be aware of this limitation, however, this was not the aim of this study, which was to quantify the hydrolysable AAs in exudates.

Quantification of clover exudates, showed that a range of exudates were produced by the plant root (Figure 3.19), with Pro exudated in the highest concentrations in the control and $^{15}NH_4{}^{15}NO_3$ application, while for $CO(^{15}NH_2)_2$ Glx occurred at the highest concentration. Plants have been shown to exudate a range of AAs (Table 3.20), and it is thought that plants are able to exudate all 20 proteinogenic AAs (Badri and Vivanco, 2009). Commonly, the studies reported in Table 3.20, regularly note that Gly and Ser are found in the greatest proportions in root exudates from various plant species (Richter et al., 1968; Ofosu-Budu et al., 1990; Jones and Darrah, 1994; Shepherd and Davies, 1994; Paynel et al., 2001a; Paynel and Cliquet, 2003; Lesuffleur et al., 2007). Paynel et al. (2001a) found that Ser and Gly were the main exudates with small amounts of other AAs, suggesting selective exudation by clover roots (*Trifolium repens*). Although findings do vary with Odunfa (1976) reporting that Glu and Ala were the most abundant AAs in Cowpea (*Vigna unguiculata*) and sorghum (*Sorghum*

bicolor), with no evidence of Pro existing in exudates. Similarly, Richter et al. (1968) noted that Pro was present in alfalfa (*Medicago sativa*) exudates but was not quantifiable, whilst in another study Ta et al. (1986) noted that alfalfa mainly released Asp, Gln, Ala and Ser. Svenningsson et al. (1990) reported Glu in the highest concentration, followed by fairly equal concentrations of Gly, Ser, and Pro in oilseed rape (*Brassica napus*). In brome grass (*Brachypodium distachyon*), 18 AAs were reported to be released from the roots with Asn being the most abundant, followed by Ser, Glu and Asp, while Pro, Cys and Trp could not be detected (Kawasaki et al., 2016). Lesuffleur et al. (2007) noted that Pro has never been observed at high concentrations in exudates despite being, along with Ser and Gly, a major proteic AA within cell walls (Showalterm 1993). Furthermore, it is commonly reported that the AAs found in high proportions in exudates, are present at low concentrations in the root extracts, it was concluded that this demonstrated that AA exudation is a selective process (Paynel et al., 2001a; Fustec et al., 2010). Nevertheless, these differ from our findings reported in Figure 3.19, showing that the relative contributions of AAs vary in exudates, showing the importance of quantifying exudates to inform the individual study.

One of the major differences between results obtained in this experiment and those reported in Table 3.20, is that the majority of these studies are reporting the free AAs within exudates, whereas AAs found within exudates from the split-root labelling experiment could have directly originated from: root exudation, protein exudation and hydrolysis by the methodology, compounds released by the roots which have undergone microbial conversions or microbes themselves (as the sand would have not remained sterile with the presence of clover roots). Furthermore, microorganisms and roots are known to compete for AAs released by the plant, plants try to minimise losses of AAs while microorganisms can release compounds which can enhance plant AA exudation (Phillips et al., 2014). The microbial community has been found to influence the AAs present in exudates, other studies have found that the concentration of Glu and Asp were greater in non-sterile clover exudates (Paynel et al., 2001b), and Bobille et al (2016) found that root exudation differs in sterilised and non-sterilised soils. Therefore, comparing plant AA exudates between different studies is extremely difficult, when a range of factors influence and compete for AAs.

Table 3.20. Different plant exudates identified and quantified in a range of different plant species

Plant species	Amino acids	Reference
Cowpea (*Vigna unguiculata*) and sorghum (*Sorghum bicolor*)	Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Val (Cys was also reported for sorghum)	Odunfa, 1976
Oilseed rape (*Brassica napus*)	Ala, Asp, Glu, Gly, Hyp, Iso, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Val	Svenningsson et al., 1990
Clover (*Trifolium repens*) and ryegrass (*Lolium perenne*)	Ala, Asn, Asp, Gln, Glu, Gly, Ser, others (not specified) (Tyr was also reported for ryegrass)	Paynel et al., 2001; Paynel and Cliquet, 2003
Alfalfa (*Medicago sativa*), medic (*Medicago truncatula*), wheat (*Triticum aestivum*), maize (*Zea mays*)	Ala, Arg, Asp, Glu, Gly, His, Iso, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val	Phillips et al., 2004
Maize (*Zea mays*), ryegrass (*Lolium perenne*), oilseed rape (*Brassica napus*), tomato (*Lycopersicon esculentum*, alfalfa (*Medicago sativa*), white clover (*Trifolium repens)*	Ala, Arg, Asn, Glu, Gln, Gly, Ser, Tyr, others (not specified)	Lesuffleur et al., 2007
Maize (*Zea mays*)	Ala, Asp, Gln, Glu, Iso, Lys, Ser Thr, Tyr	Carvalhais et al., 2010
Maize (*Zea mays*)	Ala, Asn, Asp, Gln, Glu, His+ Gly, Iso, Leu, Lys, Phe, Ser, Thr, Tyr, Val	Fan et al., 2012
Ryegrass (*Lolium perenne*)	Ala, Asp, Gly, Ser, Thr	Hertenberger and Wanek, 2014
Castor (*Ricinus communis*)	Ala, Cys, His, Gly, Lys, Met, Tyr (with Glu and Phe being found under Cu stress of 100 µmol L^{-1}, and under Cu stress of 500 and 750 µmol L^{-1} Ser and Val were present)	Huang et al., 2016
Brome grass (*Brachypodium distachyon*)	Ala, Arg, Asn, Asp, GABA, Glu, Gly + Gln, His, Iso, Leu, Lys, Met, Phe, Ser, Thr, Tyr, Val	Kawasaki et al., 2016

However, varying results between studies could be due to different methodologies used. Only some studies report that certain AAs could not be found in exudates and details are often missing for those which could not be obtained with the methodology used (e.g. Odunfa, 1976 and Kawasaki et al., 2016). The majority of studies reported in Table 3.20 used HPLC with only Svenningsson et al. (1990) reporting the use of GC-FID. Furthermore, for maize (*Zea mays*) it is noted that Pro is a known constituent of root exudates. However, it was expressed by Fan et al. (2012) that the derivatisation reagent used in their experiment reacted with primary amino groups, therefore, Pro could not be detected. Similarly, with the methods used in this project (Section 2.4), it is known that acid hydrolysis results in Asn being completely hydrolysed to Asx and Gln to Glx. Furthermore, these conditions also result in the complete destruction of Cys and Trp (Fountoulakis and Lahm, 1998; Roberts and Jones, 2008) and His (Block, 1940), along with partial reaction with Ser (~ 10% loss), Thr (~5% loss) and Tyr (although loss depends on trace levels of impurities in the hydrolysis solution) (Fountoulakis and Lahm, 1998). All methods are seen to have their drawbacks. For example, Fountoulakis and Lahm (1998) studied a range of different hydrolysis conditions with each one affecting the AAs obtained. The adopted method was chosen due to it being considered the most appropriate method for determining total AA content in soils (Roberts and Jones, 2008), which is a major part of this work. Furthermore, findings from the split-root labelling experiment showed that all AAs present within the standard AA solution (Section 2.4.2) were identified in the AA exudates. It is therefore likely that clover exudates comprise additional AAs which were not able to be identified using the adopted approach.

It is difficult to make comparisons between studies as plant exudation (both quantity and quality) is shown to be affected by a range of factors. Biotic factors which influence exudations include; root growth, type of root system, root system architecture, age of plant, plant development stage, plant species, mycorrhizal fungi, leaf and root herbivores (Hale et al., 1978; Murray et al., 1996; Bais et al., 2006; Badri and Vivanco, 2009). There are also many abiotic factors which influence plant exudations, such as light intensity, temperature, soil pH, soil solution salt and ionic concentration, particular mineral and toxic metals in the soil, soil moisture, moisture stress, oxygen concentration (hypoxia), defoliation, CO_2 enrichment, plant stress, distance between neighbouring plants (Hale et al., 1978; Ayres et al., 2007; Bazot et al., 2008; Badri and Vivanco, 2009). A range of these factors could have resulted in the differences observed between studies. Another example of differences between studies can been observed

in elicitor plant roots (molecules which stimulate defence or stress induced responses in plants) these molecules have been found to exude compounds which cannot be detected in non-elicited plants (Gleba et al., 1999). Interestingly, this study also reported a high concentration of Pro in the exudates which has not been commonly found in other studies listed in Table 3.20. Pro accumulation in plants has commonly been associated with stress (Hayat et al., 2012), which may be the reasoning for the high concentrations found in the split-root labelling experiment as the plant could be actively removing Pro accumulation. It is also surprising in the split-root labelling experiment not to see a larger quantity of Ala present within the exudates, as N_2-fixing bacteria are known to send N through the plasma membrane as Ala (Waters et al., 1998), this would suggest that active N_2-fixation was not taking place in this experiment.

Another important finding from the split-root labelling experiment was the high ^{15}N enrichment found in exudates for plants labelled with $^{15}NH_4{}^{15}NO_3$ and $CO(^{15}NH_2)_2$ (Figure 3.20). This high enrichment could have resulted from both the plant uptake of the applied ^{15}N-label and then the release of enriched exudates, as well as through microbial assimilation (introduced into the sand by root colonisation) of AA exudates released by the plant with the ^{15}N-label not taken-up by the plant. Again, findings from this experiment were in line with previous work in this chapter, showing that $CO(^{15}NH_2)_2$ results in more ^{15}N enrichment than $^{15}NH_4{}^{15}NO_3$. The findings also showed that most AA exudates were more ^{15}N enriched than the plant roots they originated from (except Gly, Hyp and Val), this could be due to the sand being sterilised with no other AAs present to dilute the ^{15}N signal as well additional incorporations of the applied ^{15}N-label which had not been taken up by the plant. This finding is interesting as studies on rhizodeposition often make the assumption that the sampled roots have the same isotopic enrichment as their deposits (Jenzen and Bruinsma, 1989; Mayer et al., 2003). However, the method used in this experiment was not necessarily the best design to study this, with the applied ^{15}N-label and exudates being within the same compartment, therefore, it could be improved by using the TC to study the exudates.

Application of $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ also exhibited a different pattern of ^{15}N enrichment of AAs, with Pro being the most ^{15}N enriched AA in the $^{15}NH_4{}^{15}NO_3$ treatment and Leu in the $CO(^{15}NH_2)_2$ treatment. This is unsurprising as the distribution of AAs was found to vary between the two treatments. In the split-root labelling experiment, the ^{15}N enrichment of individual AAs was not studied within the roots so it is hard to fully explain the results. Previously, Phe or Tyr have been shown to be the most ^{15}N enriched in the roots (comparing

^{15}N-labelling methods, Figure 3.6 and verification of leaf-labelling technique, 3.10c). However, this finding is not uncommon, some studies have shown no correlation between root and exudate AAs (Paynel et al., 2001a), while others have shown that the majority of AAs are in equal proportions in the tissues of roots and nodules compared to exudates (Ta et al., 1986). Furthermore, the results from the split-root labelling experiment do not suggest simply a ^{15}N enrichment of the AAs found in the control, as Phe and Thr were the most enriched AAs in the control exudates. Therefore, these findings suggest microbial assimilation within the sand, which is affected by the application of different ^{15}N substrates. Microbial communities are known to have a preference for certain AAs, with a particular preference shown for Asn, Arg, Gln, Glu and Lys (Shepherd and Davies, 1994a, b; Zubkov et al., 2008; Broughton et al., 2015), this could explain why some of the AAs are more ^{15}N enriched than others. This could also explain the enrichment of Pro in the $^{15}NH_4{}^{15}NO_3$ treatment, as the biosynthesis of Pro is through Glu (Nelson and Cox, 2013), coupled with the high concentration found in exudates, therefore, microbial assimilation of ^{15}N could have increased Pro enrichment. Whereas Leu is not directly synthesised from an AA source but from pyruvate, with Glu transferring the amino group in the last step of its synthesis (Binder, 2010; Nelson and Cox, 2013). Charteris (2016) also noted that when looking at the biosynthesis of applied ^{15}N-label into soil AAs, that Leu had a greater proportion of the applied ^{15}N-label than expected, concluding that there must be some preferential ^{15}N routing into Leu. These findings also suggested some preferential ^{15}N routing into Leu.

3.5.7. Summary of findings within Chapter 3

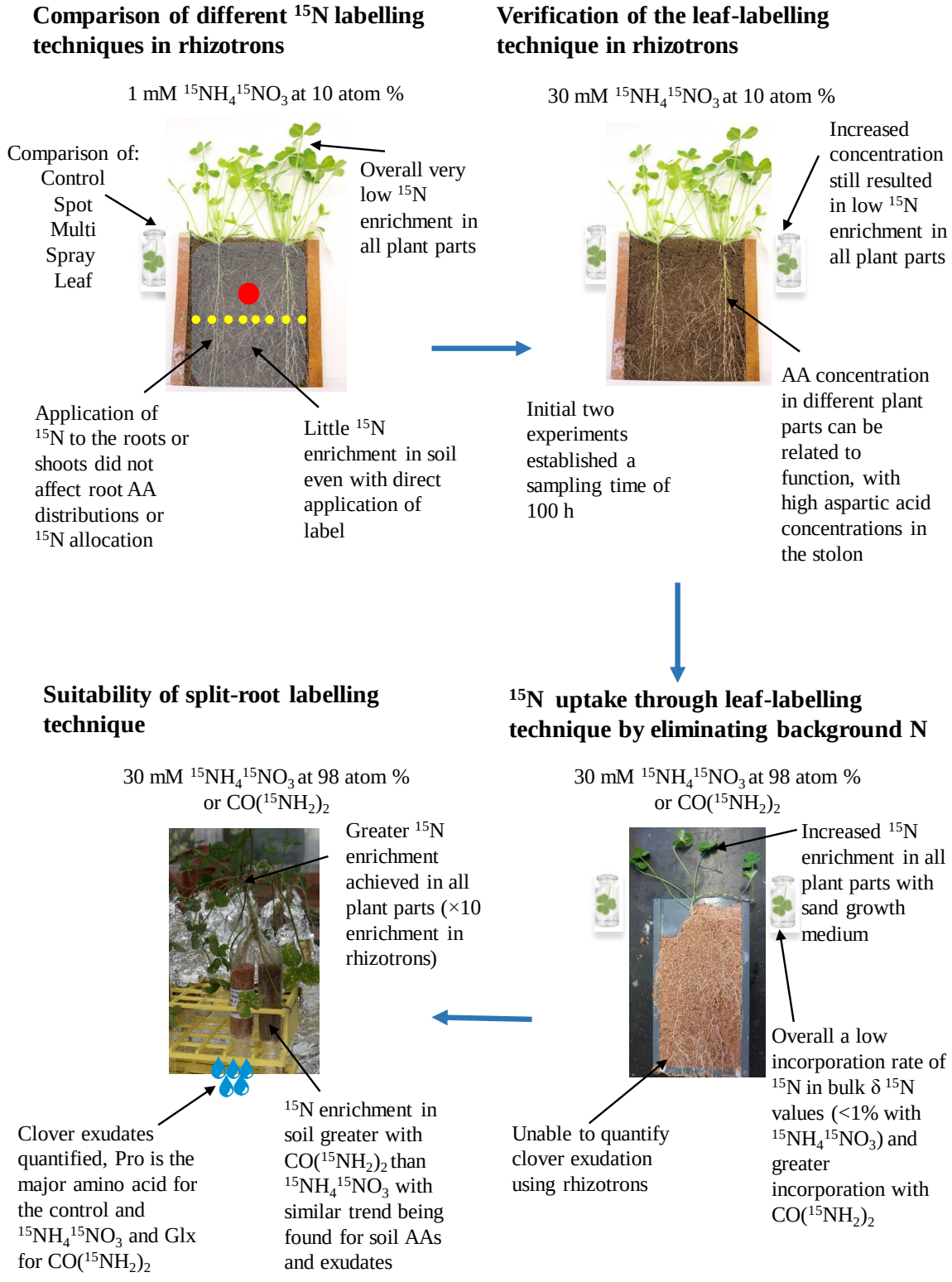


Figure 3.21. Summary figure of experiment conducted within this chapter, starting from the comparison of methods in rhizotrons, to a split-root labelling technique in incubation tubes. Experiments investigated different concentrations and atom % ^{15}N with 30 mM at 98 atom % being ideal for future experiments, and a 100 h sampling time.

3.6. Conclusion

The methods presented in this chapter will be used and further developed to provide a robust method for use in later chapters. This chapter has looked in-depth at the use of the leaf-labelling technique and a split-root labelling technique for the introduction of a ^{15}N-label to clover, with the leaf-labelling technique initially being selected due to its ease of application and field applicability. Chapter 4 will expand on these two methods to look at transfer between two plant species: clover and ryegrass.

Important specific findings relating to the objectives set out in section 3.2, include:

(i) Applying ^{15}N enriched compounds to clover plants can be done through application to the shoots or the roots. The leaf labelling technique is favourable over root labelling techniques due to a uniform distribution of the ^{15}N-label throughout all plant parts, the ease of application (especially with co-existing plant species) and its field applicability.

(ii) The application method of ^{15}N to the roots or the shoots in rhizotrons does not affect the distribution of ^{15}N in root AAs.

(iii) Monitoring the total hydrolysable AA concentrations and distributions over-time in different plant parts and soil, showed increased ^{15}N incorporation into AAs found over-time with the leaf-labelling technique. Results also revealed that the AA concentration of different plant parts can be related to their function.

(iv) Soil background N was shown to have an important role in diluting the ^{15}N in different plant parts, in order to achieve sufficient enrichment of ^{15}N within plant parts (especially great enough to see transfer between two plant species) the uptake of N from other sources (i.e. soil) needs to be minimised.

(v) Use of $CO(^{15}NH_2)_2$ as a labelling substrate was shown to be a better ^{15}N enrichment source for clover, resulting in greater ^{15}N enrichment in all plant parts than application of $^{15}NH_4{}^{15}NO_3$.

(vi) Determining AA exudation was not possible using the rhizotron technique. However, through the split-root labelling technique clover plants were shown to produce a wide range of AA exudates, with Pro being present in the greatest concentration in control plants and $^{15}NH_4{}^{15}NO_3$; for $CO(^{15}NH_2)_2$ Glx is present in the greatest concentration. Results suggested that it is likely that the presence of microbial communities influenced the release of plant exudates as well as the allocation of ^{15}N to AA exudates.

(vii) The importance of establishing the recovery rate of AAs from different plant growth mediums was shown when trying to quantify plant exudates.

(viii) AA exudates were generally more ^{15}N enriched when applying $CO(^{15}NH_2)_2$ than $^{15}NH_4{}^{15}NO_3$. A different pattern of AA enrichment was found between the two labelling substrates, for $CO(^{15}NH_2)_2$, Leu was the most ^{15}N enriched AA and for $^{15}NH_4{}^{15}NO_3$ Pro was.

(ix) Comparing the leaf-labelling and split-root labelling techniques, showed the split-root labelling technique to have far greater uptake by the plant and distribution of ^{15}N to all plant parts and into the soil.

The major implications of this chapter reveal that: in order achieve sufficient enrichment of ^{15}N within plant parts (especially great enough to see N-transfer between two plant species) a concentration of 30 mM of the labelling substrate is needed at 98 atom %, with the use of $CO(^{15}NH_2)_2$ allowing maximum enrichment. The findings also show that a sampling time of 100 h after the application of the ^{15}N-label should be used for future experiments, which would enable sufficient ^{15}N-label uptake into the plant as well as allowing the experiment to be run within a single week. The major implications of this chapter will be used within Chapter 4 to look at N-transfer between clover and ryegrass.

Chapter 4

Investigation of the routing and controls of nitrogen transfer between clover and ryegrass

4. Investigation of the routing and controls of nitrogen transfer between clover and ryegrass

4.1. Introduction

Plants deposit a significantly large amount of organic and inorganic compounds into the rhizosphere during growth, commonly referred to as rhizodeposits, they can be volatile, gaseous or non-particulate (ions, exudates, lysates, secretions) or particulate derived (border, cap, sloughed root cells and tissues, root hairs, fine and decaying roots) compounds (Wichern et al., 2008). Intercropping of legumes and non-legumes can result in the legume contributing to the N-nutrition of the non-legume (Keith et al., 1986., Janzen, 1990., Jensen, 1996a, b). N-transfer is the process of deposition by plant roots, followed by uptake by a neighbouring plant. The rhizodeposits of legumes, whether by root exudation, sloughed-off cells or dead root material, have a relatively high N-concentration, giving a low C:N ratio, indicating that N-transfer between plants is likely (Jensen, 1996b). There are a number of factors which affect the likelihood of N-transfer, such as: a rhizodeposits effect on the mineralisation-immobilisation turnover of N in soil (Robinson et al., 1989), competition between the legume and the non-legume for deposited N in the soil, capacity for N-uptake of the non-legume, and the availability of other soil N sources (Jensen, 1996b).

N-transfer between legumes and associated non-legumes has been investigated for more than 80 years by a variety of indirect (such as ^{15}N isotope dilution and the N difference method) or direct methods (such as ^{15}N shoot-labelling) (Chalk et al., 2014). The method used for determining transfer further determines whether N-transfer can be detected, if it is apparent or even real. However, quantifying transfer is inheritably difficult, as the amount of N-transferred between legume and non-legume is likely to be minimal compared to N take-up from other sources (Jensen, 1996b). Even before the development of methods which enabled transfer to be fully quantified, apparent transfer was indicated through increased productivity of the non-legume which was attributed to additional N supplied to the soil by the accompanying legume (Virtanen and Laine, 1937, Wilson and Burton, 1938). However, it is only within the last 30 years that direct methods to measure N-transfer have been described, with the development of methodologies using ^{15}N stable isotope tracers, since any tracer incorporated into a legume plant and detected in the associated non-legume plant is conclusive proof of N-transfer (Jensen, 1996b). Ledgard et al. (1985) first developed a method using foliar ^{15}N absorption to calculate N-transfer in laboratory and field experiments which enabled transfer to be calculated over a

relatively short time-scale. Using direct transfer of the ^{15}N stable isotope to calculate N flow enables some of the short-comings of the indirect ^{15}N isotope dilution and N difference methods, which only allow estimation, to be over-come. These indirect methods only allow calculation of N-transfer over long periods of time (1-5 years) and have a tendency to over-estimate N-transfer due to the 'N-sparing effect'. This is where the N_2-fixing legume roots take-up less soil N than the non-legume species and/or there is poor competition by the legume for soil N which can then be taken up by the non-legume (i.e. reduced N assimilation) (Vallis et al., 1967, Vasilas et al., 1985, Herridge et al., 1995, Chalk, 1998). Nevertheless, the use of direct methods, such as ^{15}N foliar application are not without their short falls, as they assume that applied ^{15}N, which is absorbed by the legume, will then enrich all N compounds which can be transferred. The method also assumes that any ^{15}N-label which is transferred to the soil is negligible. The method must also employ extreme care not to contaminate any soil or the non-legume with ^{15}N enriched material (Ledgard et al., 1985).

N-transfer between plant species is important for N-cycling in low-input grasslands (Høgh-Jensen and Schjoerring, 2000). However, most studies tend to focus on the N-transfer from a legume to a non-legume species, due to the capability of the legume to fix N_2 which can subsequently be transferred to the non-legume. As much as 40-50% of the non-legume N has been found to be from transfer of fixed N from legumes (Soussana and Hartwig, 1996; Høgh-Jensen and Schjoerring, 2000; Gylfadóttir et al., 2007; Rasmussen et al., 2007; Rasmussen et al., 2013). However, studies have shown that N-transfer occurs in both directions (bi-directional N-transfer) (Tomm et al., 1994; Jensen and Johansen, 1996; Høgh-Jensen and Schjoerring, 2000; Shen and Chu, 2004; Gylfadóttir et al., 2007; Rasmussen et al., 2007; Jamont et al., 2013; Rasmussen et al., 2013). The amount of N-transferred is generally less from non-legume to legume, however, there is considerable variation between estimates depending on the length of the experiment and the plant species used. Studies have shown the proportion of N-transferred from non-legume to legume to be between 5 and 8% (Høgh-Jensen and Schjoerring, 2000; Gylfadóttir et al., 2007; Rasmussen et al., 2007). However, other studies, for example, Jarmont et al. (2013), who investigated N-transfer between fababean (*Vicia faba*) and rapeseed (*Brassica napus*) and Johansen and Jensen (1996) who investigated pea (*Pisum sariuum*) and barley (*Hordeum vulgare*), found that the N-transfer was similar in both directions. However, in studies investigating N-transfer between pea (*Pisum sativum*) and barley (*Hordeum vulgare*) (Jensen, 1996b), white clover (*Trifolium repens*) and ryegrass

(*Lolium perenne*) (Paynel and Cliquet, 2003) and fababean (*Vicia faba*) and wheat (*Triticum aestivum*) (Xiao et al., 2004), no evidence of any reverse N-transfer was found.

4.2. Objectives

The work presented in this chapter builds on the methods developed in Chapter 3, which looked at methods for introducing a ^{15}N-label into a clover plant. These methods now need to be further examined for their use in estimating the N-transfer from a legume to the associated non-legume species. This chapter will provide more details and insights into the commonly used leaf-labelling technique as well as the use of the split-root. This chapter will also give details of which method will be taken forward to future chapters.

The specific objectives of this work are to:

(i) Compare uptake of ^{15}N and transfer between clover and ryegrass using the leaf-labelling and split-root technique and quantify the amount of N-transferred.

(ii) Determine if there are any physiological effects on the plant using the leaf-labelling technique, through the comparison of labelled and unlabelled substrates.

(iii) Compare the different methods for the calculation of N-transfer in plants.

(iv) Monitor the hydrolysable soil AA concentrations for response to the ^{15}N addition to clover plants.

(v) Determine and examine the patterns in individual hydrolysable soil AA $\delta^{15}N$ values in response to the addition of $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ *via* the leaf-labelling technique, and $CO(^{15}NH_2)_2$ through the split-root labelling technique.

(vi) Determine the percentages of applied ^{15}N incorporated into the total hydrolysable AA pool.

(vii) Quantify if any reverse N-transfer occurs from ryegrass-to-clover.

(viii) Determine whether AA exudation differs between clover and ryegrass.

4.3. Materials and methods

4.3.1. Transfer between plants in rhizotrons

Rhizotrons were set up as described in Section 2.2.9 before one clover cutting from the end of a stolon growing point were taken from the mother plant described in Section 2.2.5 and planted in the top of each rhizotron. Similarly, one ryegrass plant was also separated from the mother plant as described in Section 2.2.6 and placed in the same rhizotron. Rhizotrons were watered daily with a dilute ⅕ strength modified Hewitt solution and allowed to grow for six weeks in a glasshouse between July and August 2015 (see Section 2.2.5 for average, maximum and minimum temperatures). After a total of six weeks of growth, the leaf-labelling technique with two attached clover leaves was used to introduce substrates of 30 mM NH_4NO_3 ($\delta^{15}N$= 8.04 ± 0.01‰) or $^{15}NH_4{}^{15}NO_3$ at 98 atom % or $CO(NH_2)_2$ ($\delta^{15}N$= -0.09 ± 0.11‰) or $CO(^{15}NH_2)_2$ at 98 atom %, or DDW for the control. After 100 h each rhizotron was deconstructed. A soil sample was collected from each rhizotron and plant parts were separated into ryegrass and clover plants were then further divided into shoots and roots. All samples were placed in a drying oven at 80ºC for 24 h and then weighed. All samples were analysed for bulk $\delta^{13}C$ and $\delta^{15}N$ value determination (Section 2.3). In addition, AAs were also extracted from the soils, derivatised and analysed by GC-FID (quantification) and GC-C-IRMS (compound specific $\delta^{15}N$ value determination) (Section 2.4).

4.3.2. Transfer between plants in incubation tubes

Incubation tubes were set up as described in Section 2.2.10, with one tube of sand acting as the LC and two further tubes of soil for the TC and RC. Cuttings of clover and ryegrass plants were taken and allowed to grow in a pot of compost for four weeks to enable the roots to establish before inserting them into the glass Y-tubes (growing period between October and November 2016, average temperature can be found in Section 2.2.5). Clover roots were divided between the LC and TC, likewise the ryegrass roots were divided between the TC and RC. Plants in incubation tubes were moved into the contaminant section of the greenhouse, to reduce the likelihood of whitefly infestations during the running of the experiment (which were a problem in the greenhouse at the time). Plants were left to grow for a further three weeks before the experiment commenced, temperatures for the duration of the experiment can be found in Table 4.1.

After three weeks of growth, the substrates were introduced into the LC by injecting with DDW for the control or 30 mM $CO(^{15}NH_2)_2$ at 98 atom % (0.25 mL x 4) with four repeats per treatment. Incubation tubes were sampled at either 100 h or 480 h, however, controls only were sampled at 100 h. At the end of the experiment plant leaves were cut immediately to halt any further transfer, for the LC AAs were leached from the sand (with roots still intact) using 40 mL DDW. Subsequently, each incubation tube was deconstructed and plant parts and soils separated. All samples were immediately placed in the freezer, then latterly freeze-dried and then weighed. All plant and soil samples were analysed for bulk $\delta^{13}C$ and $\delta^{15}N$ value determinations (Section 2.3). In addition, AAs were also extracted from the soils and exudates, derivatised and analysed by GC-FID (quantification) and GC-C-IRMS (compound specific $\delta^{15}N$ value determination) (Section 2.4).

Table 4.1. Maximum, minimum and average temperatures (°C) in the containment section of the greenhouse during the experiment

	Max	**Min**	**Avg**
Nov-16	21.5	14.4	18.4
Dec-16	20.7	14.5	18.2
Jan-17	20.9	15.0	18.1

4.3.3. Reverse transfer between plants in incubation tubes

An identical experiment to that described in Section 4.2.3. was set up with a ryegrass plant with roots split between the LC and TC and a clover plant with roots split between the TC and RC. After taking cuttings of ryegrass and clover, plants had an initial growth period in compost of five weeks between December 2016 and January 2017 (see Section 2.2.5 for average temperatures) before being placed into incubation tubes and moved to the containment section of the greenhouse. Plants in incubation tubes were then allowed to grow for a further four weeks (average temperatures for this period are shown in Table 4.2). Subsequently, substrates were introduced into the LC by injecting with DDW for the control or 30 mM $CO(^{15}NH_2)_2$ at 98 atom % (0.25 mL x 4) with four repeats per treatment; after 100 h plant, soil and exudate samples were taken. Following the experimental period, identical steps were undertaken to sample, dry and analyse plant parts and soils as described in Sections 4.3.2.

Table 4.2. Maximum, minimum and average temperatures (°C) in the containment section of the greenhouse during the experiment

	Max	Min	Avg
Jan-17	20.9	15.0	18.1
Feb-17	21.9	16.0	18.3

4.4. Results and discussion

4.4.1. Transfer of nitrogen between plants- Leaf-labelling versus split-root

Results in this section address the objective (i) set out in section 4.2 comparing the leaf-labelling and split-root labelling techniques, in addition objective (ii) is accessed for the leaf-labelling technique only.

Immersion of clover leaves using the leaf-labelling technique developed in Chapter 3, was found to be an ineffective technique for enriching all plant parts with ^{15}N (Figure 4.1). The results show very high $\delta^{15}N$ values in the clover shoots, especially for the $CO(^{15}NH_2)_2$ (~2800‰, 1.4 atom %) compared to the $^{15}NH_4{}^{15}NO_3$ application (340‰, 0.5 atom %), but this difference was not shown to be significant. A large proportion of $CO(^{15}NH_2)_2$ was not transported into the roots, resulting in root $\delta^{15}N$ values with $CO(^{15}NH_2)_2$ and $^{15}NH_4{}^{15}NO_3$ application being similar (mean 123‰ and 140‰, respectively; $P>0.05$) (Table 4.3). Both ^{15}N enriched substrates resulted in minimal transfer to the soil and to the associated ryegrass roots. However, the $\delta^{15}N$ value of the soil is shown to be significant between treatments ($F_{4, 15}= 4.704$, $P= 0.012$), post-hoc tests show that the control is significantly different and lower than the $\delta^{15}N$ values for the $CO(NH_2)_2$, $^{15}NH_4{}^{15}NO_3$ and $CO(^{15}NH_2)_2$, but not NH_4NO_3. However, no significant difference of $\delta^{15}N$ values was observed between the ryegrass roots and the control and all other treatments. As a result, no uptake into the shoots of the ryegrass was observed (no significant difference between treatments, $p>0.05$) (Table 4.3).

One reason explaining the high levels of ^{15}N enrichment observed in the clover shoots but not being transported down into the clover roots is that plants are known to be able to store large quantities of NO_3^- in their leaves by uptake into the vacuole, where it is stored until emptied through NO_3^- assimilation (Heldt, 2005). So, by applying compounds to the shoots, this enables the plant to easily store excess N until supplies become limited and demand increases.

Furthermore, the storage of N in different plant parts depends on the number and different sinks for N present during the ^{15}N-labelling period, this is influenced by the nutritional status and the growing stage of the plant. For example, when N supply is in excess, N can be released from the xylem into plant sinks, whereas when there is a shortage in supply the reverse can take place (Götz and Herzog, 2000), this would suggest that N supply in this study was in excess causing ^{15}N to be stored in the shoot. This finding is not uncommon as previous studies using leaf-labelling have also found that the majority of ^{15}N recovered remains within the aerial parts of the plant (Zebarth et al., 1991; Rasmussen et al., 2007).

Results also show that labelling with natural abundance NH_4NO_3 and $CO(NH_2)_2$ decreased the $\delta^{15}N$ values compared to that of the control of all plant parts, especially in the ryegrass roots. However, this decrease was only found to be significantly different for the ryegrass roots ($F_{2,9}$= 6.936, P=0.015), post-hoc tests showed that the control had a significantly higher $\delta^{15}N$ value for the leaves than natural abundance NH_4NO_3 and $CO(NH_2)_2$, however natural abundance NH_4NO_3 and $CO(NH_2)_2$ did not differ from each other. The $\delta^{15}N$ values for the NH_4NO_3 labelling source were known to be 8.04‰, which is slightly higher than the $\delta^{15}N$ values of any of the control plant parts and of atmospheric air, so it is unclear why this decreased $\delta^{15}N$ values. However, for $CO(NH_2)_2$ the labelling source had a lower $\delta^{15}N$ value than the control plant parts and atmospheric air (-0.09‰), explaining the decrease seen. These results would indicate that submerging several clover leaves into concentrated substrates such as NH_4NO_3 and $CO(NH_2)_2$ does have some effect on the plant-soil system. However, no significant difference in the resulting plant biomass of all plant parts or the N content of different plant parts was found when applying any substrate (p>0.05) (Table 4.5).

Table 4.3. Statistical results for experiment looking at the leaf-labelling application to white clover (*Trifolium repens*).

ANOVA Interaction	P-value
Clover shoots: $^{15}NH_4{}^{15}NO_3$ * $CO(^{15}NH_2)_2$	NS
Clover roots: $^{15}NH_4{}^{15}NO_3$ * $CO(^{15}NH_2)_2$	NS
Soil: All treatments	P=0.012
Ryegrass roots: All treatments	NS
Ryegrass shoots: All treatments	NS
Ryegrass roots: Control * NH_4NO_3 * $CO(NH_2)_2$	P=0.015
Ryegrass shoots: Control * NH_4NO_3 * $CO(NH_2)_2$	NS

NS: main effect or interaction not significant at the P<0.05 level.

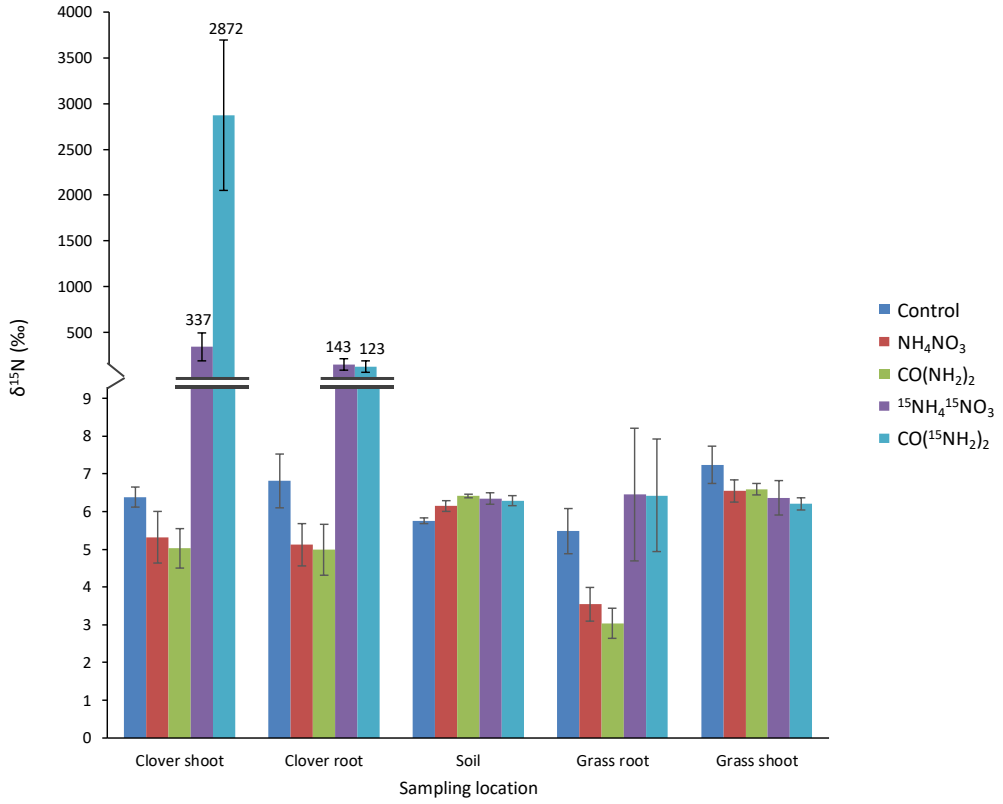


Figure 4.1. $\delta^{15}N$ values of different plant parts after application of the leaf-labelling technique to white clover (*Trifolium repens*) and determining uptake in ryegrass (*Lolium perenne*) with a 100 h labelling period (with outliers removed as described in Section 2.5.8). Leaves were either submerged in DDW for the control, natural abundance NH_4NO_3 or $CO(NH_2)_2$ or ^{15}N enriched NH_4NO_3 or $CO(NH_2)_2$. (mean ± standard error; n=3 or 4)

The split-root technique was found to be much more effective at enriching all plant parts with ^{15}N (Figure 4.2), using solely $CO(^{15}NH_2)_2$ as the enrichment substrate as this has proven to result in a much greater enrichment of plant parts than $^{15}NH_4{}^{15}NO_3$ (see Chapter 3 and Figures 3.13 and 3.16). Furthermore, the agricultural significance of $CO(^{15}NH_2)_2$ has increased, and it is now the most commonly used fertiliser worldwide (Glibert et al., 2006). The split-root labelling technique resulted in clover roots in the TC becoming notably more enriched at 100 h than the comparison leaf-labelling technique [1700 ‰ (1.0 atom %) and 120 ‰ (0.4 atom %), respectively], resulting in the observed higher ^{15}N enrichment in the associated ryegrass plant. Results in Chapter 3 also showed poor ^{15}N enrichment through the leaf-labelling technique, some studies have stated that poor recovery of ^{15}N may be due to plant losses of ^{15}N as NH_3 (de Graaf et al., 2007).

Fundamentally, the results from this experiment show that it is possible to ^{15}N-label the ryegrass shoots when the experimental period is extended to 480 h. Results typically show that when extending the experimental period from 100 h to 480 h this results in generally higher δ^{15}N values of all plant parts. However, when comparing the δ^{15}N values for the ^{15}N-labelled clover, the slight increase seen in the δ^{15}N values at 480 h compared to 100 h, is not found to be significantly different in the clover roots in the LC or TC or clover shoots (Table 4.4). As the ^{15}N moves through the plant-soil-plant system, results show a slight increase in δ^{15}N values of the soil, (control 6.5‰, 100h 11.4‰, 480h 6.8‰) showing some transfer to the soil has occurred. However, this increase is not significant when compared to the control. Similarly, there is an increased uptake of ^{15}N by the ryegrass roots in the TC (control 4.9‰, 100h 24.7‰, 480h 39.2‰), however, this increase is not found to differ significantly from the control. For the ryegrass shoots, ^{15}N uptake is only shown at 480 h, with a significant difference being found ($F_{2,9}$= 60.45, P= 0.000), where sampling at 480 h is significantly different and higher compared to the control at 100 h. For the ryegrass roots in the RC, an increase in δ^{15}N values is found for sampling at both 100 h (14.93‰) and 480 h (18.59‰) compared to the control (5.16‰), this increase is significant ($F_{2,9}$= 9.619, P= 0.006), where the control differs from both 100 h and 480 h. Finally, Figure 4.2 shows that there has been no transfer into the RC soil.

This experiment has provided fundamental information that will underpin future experiments, as it has shown that it is possible to ^{15}N-label the ryegrass shoots when the experimental period is extended to 480 h; this period will be used in later chapters. These findings also suggest that if the leaf-labelling study was extended this may substantially increase transfer into the ryegrass plant, although this does not give any suggestion as to what would happen with the distribution of the ^{15}N-label, and if the plant shoots would still retain a majority of the applied ^{15}N-label.

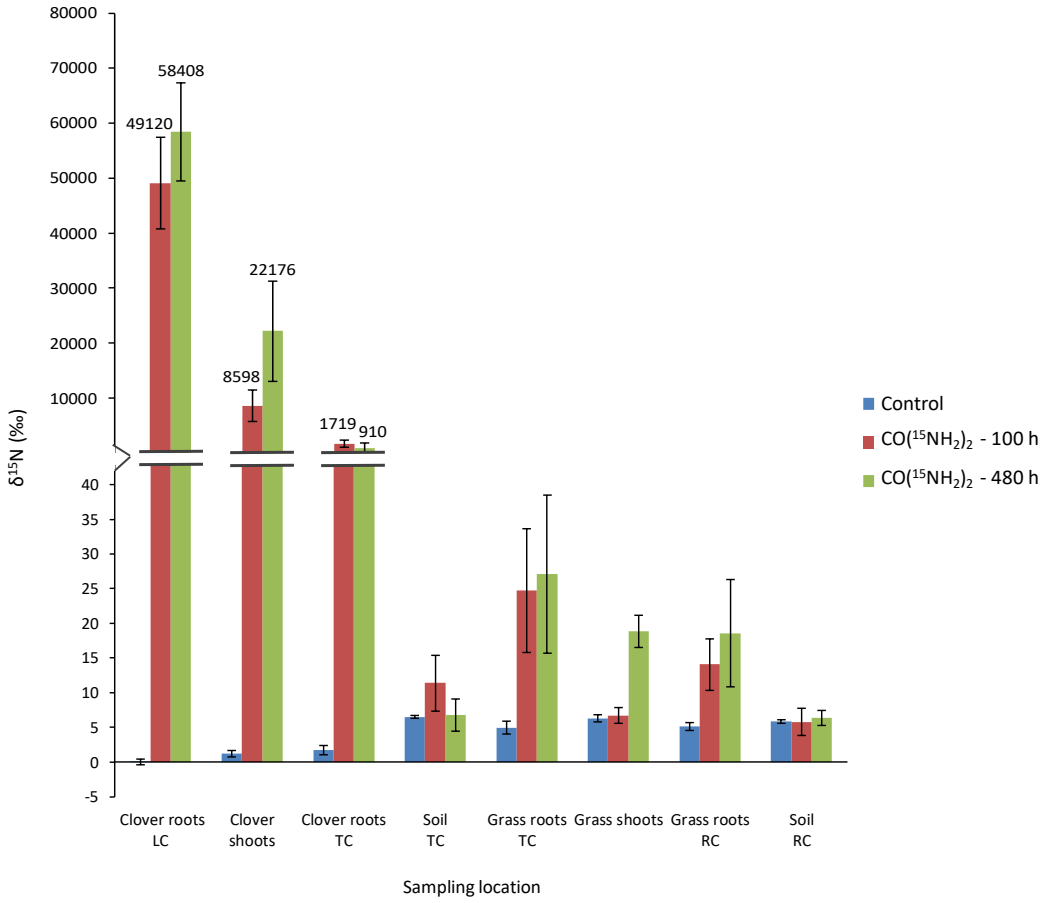


Figure 4.2. $\delta^{15}N$ values of different plant parts after application of the split-root labelling technique to white clover (*Trifolium repens*) and determining uptake in ryegrass (*Lolium perenne*) (with outliers removed as described in Section 2.5.8). Clover plants were either labelled with DDW for the control (sampled at 100 h), or ^{15}N enriched $CO(NH_2)_2$ and sampled after 100 h or 480 h. LC- labelling compartment, TC- transfer compartment and RC- receiving compartment. (mean ± standard error; n= 3 or 4).

Table 4.4. Statistical results for experiment looking at the split-root labelling application to white clover (*Trifolium repens*).

ANOVA Interaction	P-value
Clover roots LC: 100 h * 480 h	NS
Clover shoots: 100 h * 480 h	NS
Clover roots TC: 100 h * 480 h	NS
Soil: All treatments	NS
Ryegrass roots TC: All treatments	NS
Ryegrass shoots: All treatments	P=0.000
Ryegrass roots RC: All treatments	P=0.006
Soil RC: All treatments	NS

NS: main effect or interaction not significant at the P<0.05 level.

Similarly, to the leaf-labelling experiment, the application of ^{15}N enriched compounds using the split-root labelling technique had no effect on the dry matter produced by the plant or the N content compared to the control (Table 4.6) ($P>0.05$). Furthermore, it was found that extending the experimental period to 480 h, did not result in more plant biomass being produced than at 100 h. This may be due to the small growth media provided to the plants, limiting biomass production. Unsurprisingly, plants grown in rhizotrons are shown to produce more biomass than plants grown in incubation tubes, for example, clover shoot biomass for the control is 280 ± 32 mg compared to 100 ± 22 mg for rhizotrons and incubation tubes, respectively.

In comparing different labelling methods, a similar result was also found by Jensen (1996b) when comparing split-root labelling and leaf-immersion in the labelled solution [$(NH_4)_2SO_4$ with 89.8 atom % ^{15}N excess] for the donor field pea (*Pitsum sativum*) and receiver spring barley (*Hordeum vulgare L.*), also finding that the ^{15}N enrichment of the donor root with the leaf-labelling was much lower than with the split-root labelling (0.656 and 1.634 atom % ^{15}N, respectively) with similar values being found in the shoots. Furthermore, Jensen also showed more N-transfer using a split-root technique than with leaf-labelling, which further supports our findings (Section 4.4.2).

For the results shown in Figure 4.2, outliers have been removed (as described in Section 2.5.8), original results for the $CO(^{15}NH_2)_2$ treatment at 100 h and 480 h showed one sample for each treatment being much more highly enriched with ^{15}N than the rest of the repeats. Samples which have been removed from the averages, in particular, exhibited high enrichment in the TC soil resulting in high enrichment in the ryegrass roots, however, this did not necessarily result in high ^{15}N enrichment in the rest of the plant-soil system (i.e. ryegrass shoots, RC roots or RC soil). These results further showed an uneven flow of N, as it would normally be assumed that higher ^{15}N enrichment in transferring plant parts or soils would result in an effect being seen through the whole plant system.

Table 4.5. Dry matter and N-content for plant parts sampled after a 100 h leaf-labelling applying either DDW (control), natural abundance NH_4NO_3 or $CO(NH_2)_2$ or ^{15}N enriched NH_4NO_3 and $CO(NH_2)_2$ to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*) (mean ± standard error; n=4). One-way ANOVA result comparing the effect of labelling substrate on the resultant plant dry matter and N content.

	Dry matter (mg plant^{-1})				N content (mg plant^{-1})			
	CLOVER		RYEGRASS		CLOVER		RYEGRASS	
	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots
Control	279 ± 31.9	79 ± 8.9	510 ± 69.4	70 ± 21.8	10.0 ± 1.0	2.2 ± 0.1	18.9 ± 2.3	1.6 ± 0.4
NH_4NO_3	184 ± 28.4	71 ± 14.5	547 ± 38.1	66 ± 10.9	6.1 ± 0.8	1.8 ± 0.4	20.1 ± 1.0	1.4 ±0.2
$CO(NH_2)_2$	201 ± 40.2	104 ± 12.5	594 ± 72.6	84 ± 17.9	7.0 ± 1.5	2.4 ± 0.3	21.6 ±2.4	1.6 ± 0.4
$^{15}NH_4{}^{15}NO_3$	265 ± 28.0	99 ± 15.1	413 ± 80.5	61 ± 13.5	8.8 ± 1.3	2.8 ± 0.3	15.2 ± 2.5	1.1 ± 0.2
$CO(^{15}NH_2)_2$	288 ± 64.0	102 ± 19.5	327 ± 112.0	43 ± 8.9	10.5 ± 2.7	2.8 ± 0.5	11.2 ± 4.2	0.9 ± 0.2
ANOVA	NS	NS	NS	NS	NS	NS	NS	NS

NS: main effect or interaction not significant at the $P<0.05$ level.

Table 4.6. Dry matter and N content for plant parts sampled after a 100 h and 480 h split-root labelling with ^{15}N enriched NH_4NO_3 to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*) (control received DDW and sampled at 100h). LC= labelling compartment, TC= receiving compartment, and RC= receiving compartment (mean ± standard error; n=4). One-way ANOVA result comparing the effect of time and labelling substrate on the resultant plant dry matter and N content.

	Dry matter (mg plant^{-1})					
	CLOVER			RYEGRASS		
	Roots LC	Shoots	Roots TC	Roots TC	Shoots	Roots RC
Control	20 ± 3.2	101 ± 21.6	22 ± 7.7	35 ± 8.3	344 ±56.7	38 ± 8.2
100 h	39 ± 4.4	190 ± 18.5	38 ± 7.0	19 ± 12.8	317 ± 21.3	40 ± 7.8
480 h	24 ± 8.0	153 ± 12.4	29 ± 8.2	39 ± 8.2	464 ± 77.6	34 ± 10.7
ANOVA	NS	NS	NS	NS	NS	NS
	N content (mg plant-1)					
Control	0.56 ± 0.08	3.92 ± 0.78	0.51 ± 0.18	0.57 ± 0.10	10.5 ± 1.46	0.63 ± 0.13
100 h	0.98 ± 0.08	6.55 ± 0.59	1.02 ± 0.16	0.37 ± 0.21	9.76 ± 1.32	0.61 ± 0.10
480 h	0.83 ± 0.19	5.54 ± 1.21	0.79 ± 0.3	0.51 ± 0.12	13.0 ± 2.39	0.48 ± 0.13
ANOVA	NS	NS	NS	NS	NS	NS

NS: main effect or interaction not significant at the $P<0.05$ level.

One of the primary objectives of this thesis is to study the routing and controls on N-transfer between plants, to do so it is necessary to study the processes that are occurring in the soil which is where transfer fundamentally occurs. Results in Figures 4.1 and 4.2, show very little increase of $\delta^{15}N$ values in the soil, meaning that only a small proportion of the ^{15}N-label applied to the clover plant is incorporated into the bulk soil, this is further discussed in Section 4.4.4. Despite the results being very small it shows that some of the ^{15}N-label applied to the plant, either through leaf or split-root labelling, is incorporated into the bulk soil N pool (Table 4.7). No difference is found between incorporation rates into the bulk soil using $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ through leaf-labelling, or in the split root labelling technique at 100 h and 480 h.

Table 4.7. Incorporation of applied ^{15}N-label through leaf and split-root labelling into bulk soil $\delta^{15}N$ values (%) in the two different experiments to measure transfer. One-way ANOVA result comparing the incorporation of ^{15}N-label into the bulk soil with the leaf-labelling and split-root labelling techniques.

	Incorporation of ^{15}N-label into bulk soil (%)
Transfer with leaf-labelling technique in rhizotrons	
$^{15}NH_4{}^{15}NO_3$	0.020 ± 0.005
$CO(^{15}NH_2)_2$	0.018 ± 0.004
AVONA	NS
Transfer with split root-labelling technique in culture tubes	
100 h	0.156 ± 0.072
480 h	0.010 ± 0.010
AVONA	NS

NS: main effect or interaction not significant at the $P<0.05$ level.

4.4.2. Calculating transfer between plants

Results in this section address the objective (iii) as set out in section 4.2, as previously detailed in the Section 2.5.6, there are many different expressions available to calculate the N-transfer between plants using ^{15}N-labelling techniques. These are compared in Table 4.8 for the leaf and split-root labelling techniques. Results show that no N-transfer between clover and ryegrass was detected using the leaf-labelling technique with $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$, this result further supports the findings displayed in Figure 4.1, which show very little ^{15}N enrichment of the associated non-legume ryegrass plant. For the split-root labelling technique, the proportion of non-legume N derived from the transfer of legume N (Ndft) was greater at 480 h after labelling, varying from 2.17% to 5.01% depending on the expression used, and for 100 h from 0.169% to 0.558%. Despite this, no significant difference was found between N transfer at 100 h and 480 h using any of the expressions ($P>0.05$). Typically, an increase in N-transfer with time is found and has been shown in other studies, such as, in Jensen (1996b) concluding that this is probably due to the accelerated turn-over from the root and nodules. Yield-dependent and independent expressions which Ndft tended to be in agreement with each other and similarly for expressions calculating proportion of N in the non-legume derived from the transfer of legume root N ($Ndft_r$). However, Ndft and $Ndft_r$ estimates differed from each other.

There have been a number of reviews undertaken to compare the different yield-dependent and independent expressions available in the literature, such as Chalk and Smith, 1997 and Chalk et al., 2014. It is likely that the equation used needs to take into the account the results obtained by the experimental procedure. For example, the majority of expressions assume that transfer to the soil N pool is non-existant, however, a number of studies have shown that N-transfer to the soil N pool can be considerable (Ross et al., 1964., Poth et al., 1986., McNeill et al., 1997,1998., Khan et al., 2002b; Chalk et al., 2002). Results for these experiments also suggest some ^{15}N-transfer to the soil, although varying with the experiment undertaken. For the leaf-labelling technique estimates for transfer to the soil N pool are 0.007% and 0.005% for $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$, respectively (Figure 4.1), and for the split-root labelling technique 0.392% and 0.254% for 100 h and 480 h after labelling, respectively (Figure 4.2). The variation between these two experiments shows that transfer to the soil should be established before the appropriate expression is chosen, and needs to be considered for the split-root labelling technique in particular. Calculations which do not take into account substantial transfer to the soil, will result in the overestimation of N-transferred between plants.

Furthermore, on choosing the appropriate expression, Giller et al. (1991) proposed that ^{15}N enrichment of the legume root at harvest may better represent the ^{15}N enrichment of the N transferred ($Ndft_r$). This is particularly important in expressions that calculate transfer as results show, in Figure 4.1 and Figure 4.2, that the legume which donates its N to the non-legume is uniformly labelled. Results from this study further show that the plant part which receives the ^{15}N-label (root or shoot) is vastly more ^{15}N enriched that the rest of the plant, revealing poor transfer and partitioning of ^{15}N throughout the plant. However, when initially studying the leaf-labelling technique (Chapter 3), a uniform distribution of the label was found in different plant parts, this is likely to be due to the low concentration in the initial experiments, and then the use of sand in equivalent concentrations and atom % to these experiments minimalizing any initial uptake of N, resulting in ^{15}N being transported to the roots. The results in this chapter further support the notion that ^{15}N enrichment of the legume root better represents the ^{15}N enrichment of the N-transferred ($Ndft_r$) and expressions which use this should be adopted. The non-uniform distribution of the ^{15}N-label has been shown in a number of studies (Ledgard et al., 1985, Giller et al., 1991, Khan et al., 2002a, b) with Johansen and Jensen (1996) agreeing that the use of root N enrichment is more meaningful. However, Chalk and Smith (1997) expressed concern over using the weighed mean ^{15}N enrichment of all plant parts over the root ^{15}N enrichment at harvest as they found no consistent relationship between the two, with estimates hugely varying depending on which method was used. They also concluded that these estimates are unlikely to truly represent the ^{15}N enrichment of N-transfer and the decline in ^{15}N enrichment of the plant over-time should be considered. This is particularly important with foliar labelling, which in Chapter 3 was shown to reach a critical point before resulting in declining ^{15}N enrichment. However, the leaf-labelling technique here is shown to be ineffective for studying N-transfer between plants, consequently, the split-root labelling technique will be used in future studies. This study compared plant harvesting after 100 h and 480 h, showing increased N-transfer with time, and therefore declining ^{15}N enrichment is not a concerning factor in calculating transfer in later chapters.

For the purpose of this work, Equation 2.26 will be used in all further chapters to calculate transfer between plants and compare treatments as it takes into account transfer of N to the soil as well as the non-uniform distribution of the ^{15}N-label.

Notwithstanding the variation seen for estimating N-transfer between plants, all estimates showed very low amounts of N being transferred, especially compared to other studies (such as: Soussana and Hartwig, 1996; Høgh-Jensen and Schjoerring, 2000; Gylfadóttir et al., 2007; Rasmussen et al., 2007; Rasmussen et al., 2013). The low estimates are surprising as white clover is often found to donate the most N to neighbouring plant species when compared to other forage legumes (Pirhofer-Walzl et al., 2012, Rasmussen et al., 2012), which would suggest high amounts of transfer should have occurred in this study. Results obtained for this work could be low because transfer was only studied for a relatively short term (100-480 h equivalent to 4-20 days), compared to growing seasons typically reported in the field studies (Høgh-Jensen and Schjoerring, 2000; Glyfadóttir et al., 2007; Rasmussen et al., 2007), however, transfer was often noted to be apparent in the first 20 days. Rasmussen et al. (2013) noted that short-term transfer of ^{15}N in white clover-to-ryegrass was greater than 50% in the late growing season (three-week period), questioning the role that longer-term root turnover processes have in N-transfer. However, other studies have shown that the amount of N-transfer varies throughout the growing season (Høgh-Jensen and Schjoerring, 2000; Rasmussen et al., 2013). As well as N-transfer increasing over the longer term, this is particularly important as pastures are often managed on a perennial or semi-perennial basis (Høgh-Jensen and Schjoerring, 2000). Over a three-year study period, Burity et al. (1989), Høgh-Jensen and Schjoerring (1997) and Jørgensen et al. (1999) all showed that transfer increased with the age of the sward (16%, 3%, and 3% in the first year (seedling year) to 36%, 22% and 34%, respectively, in the third production year). Neither of these factors were able to be replicated by the laboratory experiment, as plant growth could not be supported for a longer duration. Therefore, the young plants used in these experiments may have not been established well enough for effective N-transfer. Furthermore, restrictions could have been induced on the amount of N-transferred due to the restriction of normal root growth, development and structure by the experimental conditions, this would affect estimates compared to field experiments. However, similar transfer was reported by Ledgard et al. (1985) who grew plants in pots [2.2% N transferred from subterranean clover-to-ryegrass (*Trifolium subterraneum* and *Lolium rigidum*) over 29-days], with no measurable transfer identified in a 36-day field experiment. Similarly, Morris et al. (1990) found no significant N-transfer over an entire season from arrowleaf clover (*Trifolium vesiculosum*) to ryegrass (*Lolium multiflorum*). The experiment conducted in this chapter did not look at quantifying biological N_2-fixation by clover, therefore, we do not know if clover was actively fixing N_2. The soil used in this experiment was not of low N status (0.56% N), making N_2-fixation more unlikely, due to the

preferential uptake of soil N compared to the high energy demanding process of N_2-fixation. Furthermore, although low (30 mM) clover did receive an input of $CO(^{15}NH_2)_2$ to ^{15}N enrich the plant parts to study transfer, which could have affected the dynamics of N_2-fixation and transfer. When N fertilisation is applied to co-existing clover and ryegrass roots, it has been shown to reduce N-transfer from clover-to-ryegrass (Rasmussen et al. 2013). In other ^{15}N-labelling studies it has been found that large amounts of N-transferred between plants has been unlabelled N (from fixation) complicating efforts to quantify N-transfer (Murray and Hatch, 1994), this could have similarly effected quantification of N-transfer in this experiment. Additionally, the N-transfer is often seen to be greatest in low N status soils, with only minimal direct N-transfer with greater soil N availability (Murray and Clements, 1998) and is often found to be driven by a source and sink relationship between plants (Frey and Schüepp, 1992), therefore, ryegrass demand simply could have been low. Finally, the levels of N-transfer could be seen to be low due to any deposited N being subjected to re-uptake by clover. Rasmussen et al. (2013) found that both ryegrass and clover re-uptake deposited N, which corresponded to at least 1% of N in the leaf biomass. It is unlikely that N was lost out from the system as effort was made to minimise the amount of leaching.

Table 4.8. Comparison of expressions available to estimate the proportion of non-legume N derived from the transfer of legume N [for white clover (*Trifolium repens*) to associated ryegrass (*Lolium perenne*)] in percentage (%). Expressions are detailed in Section 2.5.6 (mean ± standard error; n=3 or 4). One-way ANOVA result comparing N-transfer with the split-root labelling technique at 100 and 480 h.

	Yield dependent calculations				Yield independent calculations	
	Ndft Equation 2.23	$Ndft_r$ Equation 2.24	Ndft Equation 2.25	$Ndft_r$ Equation 2.26	Ndft Equation 2.27	$Ndft_r$ Equation 2.28
Transfer with leaf-labelling technique in rhizotrons						
$^{15}NH_4{}^{15}NO_3$	-	-	-	-	-	-
$CO(^{15}NH_2)_2$	-	-	-	-	-	-
Transfer with split root-labelling technique in culture tubes						
100 h	0.169 ± 0.079	0.504 ± 0.209	0.225 ± 0.075	0.558 ± 0.183	0.169 ± 0.079	0.539 ± 0.224
480 h	2.17 ± 1.63	2.49 ± 0.818	2.17 ± 1.63	2.24 ± 0.735	2.59 ± 2.03	5.01 ± 2.01
ANOVA	NS	NS	NS	NS	NS	NS

NS: main effect or interaction not significant at the $P<0.05$ level.

4.4.3. Effect of different labelling techniques on soil amino

The quantity of hydrolysable soil AAs is shown to differ in the two experiments, with a higher quantity of soil AAs being found with the leaf-labelling technique which was conducted in rhizotrons with 100 g of soil. The higher volume of soil in the rhizotrons, compared to 15 g in the incubation tubes used for the split-root labelling technique, allowed higher amounts of plant biomass to be produced (Table 4.5 and 4.6), especially in the roots, thereby accounting for this difference.

Between the two different labelling techniques (4.3a and b), the pattern of distribution of the soil AAs varies slightly, for example, Glx is clearly the most concentrated AA for control soils in the leaf-labelling technique but not for the split-root labelling technique where the most concentrated AA is Pro. However, the most notable difference concerns Lys which is shown to be at a much higher concentration in soil using the split-root labelling technique than the leaf-labelling. It is hard to directly compare these two experiments as they were not conducted simultaneously and had different initial growing periods and temperatures. Temperature differences could have caused this effect, causing overall different plant growth rates as well as influencing the soil microbial community which is responsible for much of the biosynthesis of AAs in the soil. Although turn-over and synthesis of AAs in the soil is seen to occur rapidly, these results could have been due to different metabolic processes occurring in each soil, resulting in differences in soil AAs between these two experiments. The N transfer metabolic pathway for Lys proceeds from Asp which is one of the major AAs found in the soil, suggesting different processes are occurring at the sampling time of these two experiments.

The major finding in Figure 4.3, is the effect that leaf-labelling is shown to have on the soil AAs (and is not observed with the split-root labelling technique). Results show that when leaf-labelling with highly enriched ^{15}N compounds ($^{15}NH_4{}^{15}NO_3$ and $CO(^{15}NH_2)_2$) the concentration of soil AAs sustainably and significantly decreases over the whole range of AAs studied compared to the control and soils subject to application of natural abundance substrates. As a result a significant decrease is seen in the total hydrolysable AA content by almost 50% for the soils subjected to $^{15}NH_4{}^{15}NO_3$ and $CO(^{15}NH_2)_2$ application compared to the control (Table 4.9) ($F_{4,15}$=20.738, P=0.000). This finding has not been shown in any other studies, and further supports the findings in Figure 4.1 that submerging plant leaves in a concentrated solution does have some, detrimental, effect on the plant-soil system. It is known that foliar application (mist

or spray) of $CO(NH_2)_2$ can result in crop leaf damage (Bremner, 1995; Gooding and Davies, 1992), therefore, concentrations of $CO(NH_2)_2$ are often kept low in studies (Schmidt and Scrimgeour, 2001). However, comparatively low concentrations were used in this study and leaf damage was not evident.

A common assumption made when using the leaf-labelling technique is that applying ^{15}N compounds to the plant shoots does not cause any modifications to the root N dynamics, function or behaviour (Chalk et al., 2014). Some concerns have been expressed that artefacts may be introduced when loading the plant xylem and phloem with greater N concentrations than normal, causing increased root growth, turnover or exudation (Chalk et al., 2014). Høgh-Jensen and Schjoerring (2000) expressed that the application of N compounds through the leaf will inevitably have an effect on the internal leaf metabolism, but concluded this would most likely lead to exudation of ^{15}N enriched compounds from the roots. Similarly, Rasmussen et al. (2013) concluded that observations of high transfer rates from clover-to-ryegrass might be due to leaf-labelling resulting in higher exudation of N although only in the short term, due to uptake of N being through normal routes. However, neither study provided evidence to support these claims. This study did not show excessive root growth (Table 4.5), and no change in the total C and N dynamics of the soil (Table 4.9, C content varied between 4.23- 5.26% and N content between 0.438-0.549% for all samples, $P>0.05$), although this change may be too small to be detected in the overall bulk soil. Thorne (1957) found that leaf-labelling sugar beet with $^{15}NH_4NO_3$ resulted in an increased uptake of unlabelled soil N by the roots compared to the unlabelled control, however, this increased uptake was also coupled with increased plant biomass. Similarly, Sen and Chalk (1996) showed that a plant's response to foliar application of $CO(^{15}NH_2)_2$ depended on the soil N status, where both sunflower and wheat were found to take up considerably higher amounts of unlabelled soil N in lower N fertility soils compared to the control, however this result depended on the number of $CO(^{15}NH_2)_2$ applications to the leaves. Fundamentally, leaf-labelling does not follow the natural pathway of N assimilation.

Returning to the results in Chapter 3, the comparing ^{15}N-labelling methods experiment also showed a slight decrease in the concentration of root AAs using the leaf-labelling method compared to the control (Figure 3.6). This decrease is nowhere near as pronounced as the findings in Figure 4.3b, but these experiments also used a low concentration of $^{15}NH_4{}^{15}NO_3$ (1 mM at 10 atom % compared to 30 mM at 98 atom %). Furthermore, it should be noted, although no effects on the soil AA concentration were observed using the split-root labelling technique

results from Chapter 3, looking at clover exudation (Figure 3.19), showed a high concentration of Pro which may be as a result of plant stress in control and $^{15}NH_4{}^{15}NO_3$ plants. Although, no significant difference was found, the total hydrolysable AA content for the $CO(^{15}NH_2)_2$ was less than the control or $^{15}NH_4{}^{15}NO_3$ (averaging 0.23 mg, 0.28 mg and 0.33 mg, respectively). Therefore, it could be concluded that application of any compounds highly enriched in ^{15}N have an effect on the plant system.

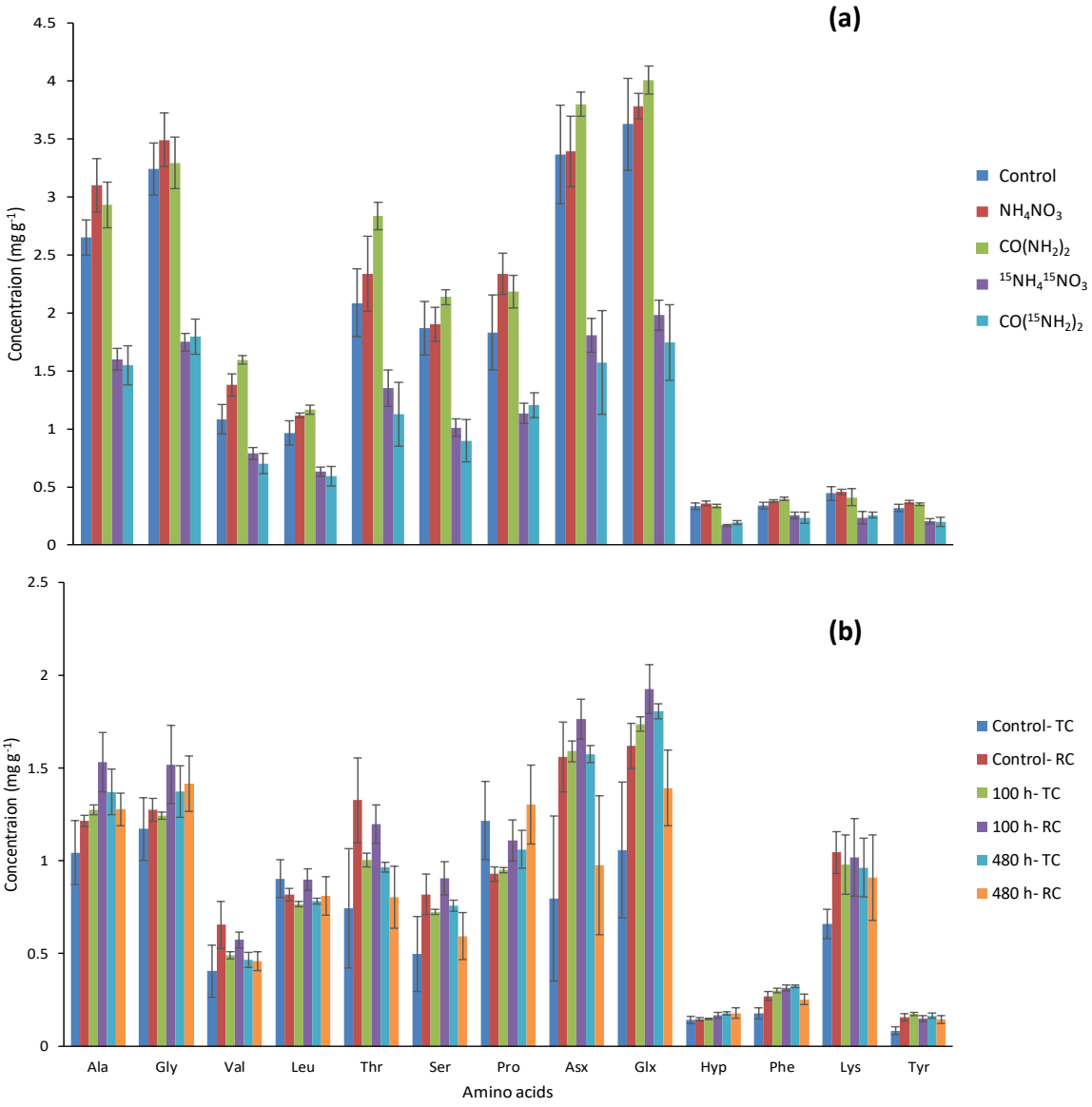


Figure 4.3. Concentration of AAs [mg of AA per gram of sample (mg g^{-1})] in soil after application of ^{15}N-label to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*): (a) leaf-labelling technique. Leaves were either submerged in DDW for the control, natural abundance NH_4NO_3 or $CO(NH_2)_2$ or ^{15}N enriched $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ and harvested after 100 h. (b) Split-root labelling technique with DDW for the control (sampled at 100 h), or ^{15}N enriched $CO(NH_2)_2$ and sampled after 100 h or 480 h. TC- transfer compartment and RC- receiving compartment. (mean ± standard error; n= 4)

Unlike the leaf-labelling approach, the split-root labelling technique showed no effect on the concentration of individual AAs (Figure 4.3b) and therefore the resultant hydrolysable AA content was consistent between different treatment applications and sampling locations ($P>0.05$). As expected, there was no variation in the total C and N content (Table 4.9) for the split-root labelling technique and similar values were found using both techniques.

Table 4.9. Mean soil total N (% TN), soil total C (%TC), total soil hydrolysable AA content and total soil hydrolysable content which is N (mg g^{-1}) for the leaf-labelling technique and the split-root labelling technique. TC- transfer compartment and RC- receiving compartment. One-way ANOVA result comparing the %TN, %TC and total hydrolysable amino acid content in the soil with the leaf-labelling and split-root labelling technique.

	%TN	%TC	Total hydrolysable amino acid (mg g^{-1})	Total hydrolysable amino acid N (mg g^{-1})
Leaf-labelling				
Control	0.500	4.73	22.16	2.92
NH_4NO_3	0.532	5.03	24.4	3.20
$CO(NH_2)_2$	0.522	5.05	25.44	3.30
$^{15}NH_4{}^{15}NO_3$	0.515	4.92	12.92	1.68
$CO(^{15}NH_2)_2$	0.503	4.68	12.07	1.59
ANOVA	NS	NS	P=0.000	-
Split-root labelling				
Control- TC	0.505	4.89	8.89	1.19
Control- RC	0.503	4.85	11.82	1.57
100 h- TC	0.490	4.87	11.37	1.51
100 h- RC	0.503	4.90	13.07	1.74
480 h- TC	0.494	4.85	11.78	1.56
480 h- RC	0.502	4.98	10.5	1.42
ANOVA	NS	NS	NS	-

NS: main effect or interaction not significant at the $P<0.05$ level.

4.4.4. Incorporation of ^{15}N-labelled substrates in soil amino

Results in this section address the objectives (iv), (v) and (vi) set out in section 3.2. As shown in Figures 4.1 and 4.2 there was very little increase of bulk δ^{15}N values in the soils, especially for the leaf-labelling study. Figure 4.4a further confirms that there was little assimilation by the soil microbial biomass of the ^{15}N-label into individual AAs, with all applied substrates (natural abundance or ^{15}N enriched) showing similar δ^{15}N values. These results make it difficult to comment on any processes and N-cycling occurring in the soil. However, treatments of $^{15}NH_4{}^{15}NO_3$ and $CO(^{15}NH_2)_2$ show Ser and Tyr to have very slightly elevated δ^{15}N values compared to the control (although the change is not significantly different $P>0.05$). Most likely, this slight shift is due to instrumental error as it lies within ±1‰. The leaf-labelling technique has been shown to have an effect on plant physiology, which in turn has been shown to affect the distribution of soil AAs (Figure 4.3a), as a result it is also likely to affect the distribution of ^{15}N in soil.

For the split-root labelling study, the bulk δ^{15}N values (Figure 4.2) were shown to be elevated in the TC soil at 100 h and 480 h (11.38‰ and 6.79‰ respectively, compared to the control 6.49‰, although not significantly), however, this increase is not so apparent in the whole spectrum of AAs. The low increase of δ^{15}N values for the bulk soil and individual soil AAs may simply arise from plant uptake of any ^{15}N exudate by plant roots (re-uptake by clover or release by clover and taken up by ryegrass). Plants were grown in relatively small containers of soil with no additional N inputs, therefore plant uptake of soil N both in the organic and inorganic forms is likely. This also suggests that the plants out-competed the soil bacteria for any ^{15}N released, as otherwise larger incorporations into the AAs would have been observed. Alternatively, N was transferred directly between plants by mycorrhizal fungi, the relevance of which has been investigated in several previous studies (e.g. Haystead et al., 1988; Barea et al., 1989a; Hamel et al., 1991a, b, c; Ikram et al., 1994). As previously discussed (Section 4.4.4) when calculating N-transfer between plants, it is often assumed that little or no transfer occurs to the soil N pool, bulk results from this study showed transfer to the soil definitely occurred (although in small amounts), emphasising the need to couple compound-specific isotope work with bulk isotope results.

For the split-root labelling study (Figure 4.4b), Asx and Glx are shown to have slightly elevated δ^{15}N values in the TC at 100 h and 480 h compared to the control (although a significant

difference is only found for Asx, $F_{2,7}$= 7.304, P=0.019, where 100 h is significantly higher than the control or 480 h). Results for the split-root labelling study (Figure 4.4b) suggest that assimilation into AAs has only just commenced, and N has not been transferred to the full spectrum of AAs. Previous studies have shown that larger quantities of NH_4^+ are released from plants than AAs (Paynel et al., 2001a; Paynel and Cliquet, 2003). Therefore, the most likely explanation for the increase is that, NH_4^+ released is synthesised into AAs, with biosynthesis playing a key role in the shift in $\delta^{15}N$ values of individual AAs. These two AAs (particularly Glx) are an important starting point in the biosynthesis of other AAs, playing an important role in N transfer to other AAs. Glu is used to synthesis new AAs, where it is the C skeletons for new AAs (Section 1.3.1). Glu coupled with oxaloacetate forms Asp (Berg et al., 2015), which is then the main precursor for Met, Thr, Iso and Lys.

Previous studies have shown different incorporation rates into soil AAs through the addition of ^{15}N, for example, in a timed series experiment (32 days), Charteris et al. (2016) showed that $^{15}NH_4$ is incorporated into Glx more quickly over the first 2 days of the experiment than any other AA with a two to five-fold ^{15}N enrichment, before declining for the remainder of the experimental period as a result of the redistribution of ^{15}N into newly synthesised AAs. They showed that the differences in patterns of ^{15}N incorporation may be related to established biosynthetic pathways. However, this experiment does not enable in-depth study into the synthesis of AAs over time since only two time points have been studied and for the majority of AAs there is no change in ^{15}N incorporation over-time.

Furthermore, the results in this chapter (increased $\delta^{15}N$ values of Glx and Asp) are most likely to result from AA assimilation and not exudates. As this would be likely to result in an increase in $\delta^{15}N$ values of a wider range of AAs, especially for 480 h where fairly equal quantities of individual AAs are shown to be exudated (Figure 4.8). However, elevated soil AA $\delta^{15}N$ values could be attributed to root material not being recovered (full recovery is near impossible). The major AA in clover roots is Asn (Paynel et al., 2001a) and in the AA protocol used it is converted completely to Asx (Fountoulakis and Lahm, 1998). Whereas, for ryegrass roots the major constituent AA are Gln and Glu (again Gln is converted to Glx during hydrolysis). However, if large quantities of roots still remained in the soil then this would have most likely resulted in the $\delta^{15}N$ value being much greater than seen here.

For the split-root labelling technique, Pro is found to have significantly elevated $\delta^{15}N$ values in the TC at 100 h compared to the control and 480 h ($F_{2,7}$= 235.7, P=0.000). It is unclear why this has happened, however, plant stress has been shown to result in Pro accumulation, which may have occurred under experimental conditions, altering AA exudation (Hayat et al., 2012). Previous experimental work (Figure 3.18) and results presented in this chapter (Figure 4.8) have shown that a large amount of Pro is exudated by clover roots compared to other AAs. However, the concentration of Pro in the soil was unaffected (Figure 4.3b), although it is likely that much greater amounts of Pro would need to be exuded to alter the overall concentration in the bulk soil. In addition, proline is synthesised from Glu, so it could have been biosynthesised (Nelson and Cox, 2013), although it is still not clear why this process would also not occur at 480 h.

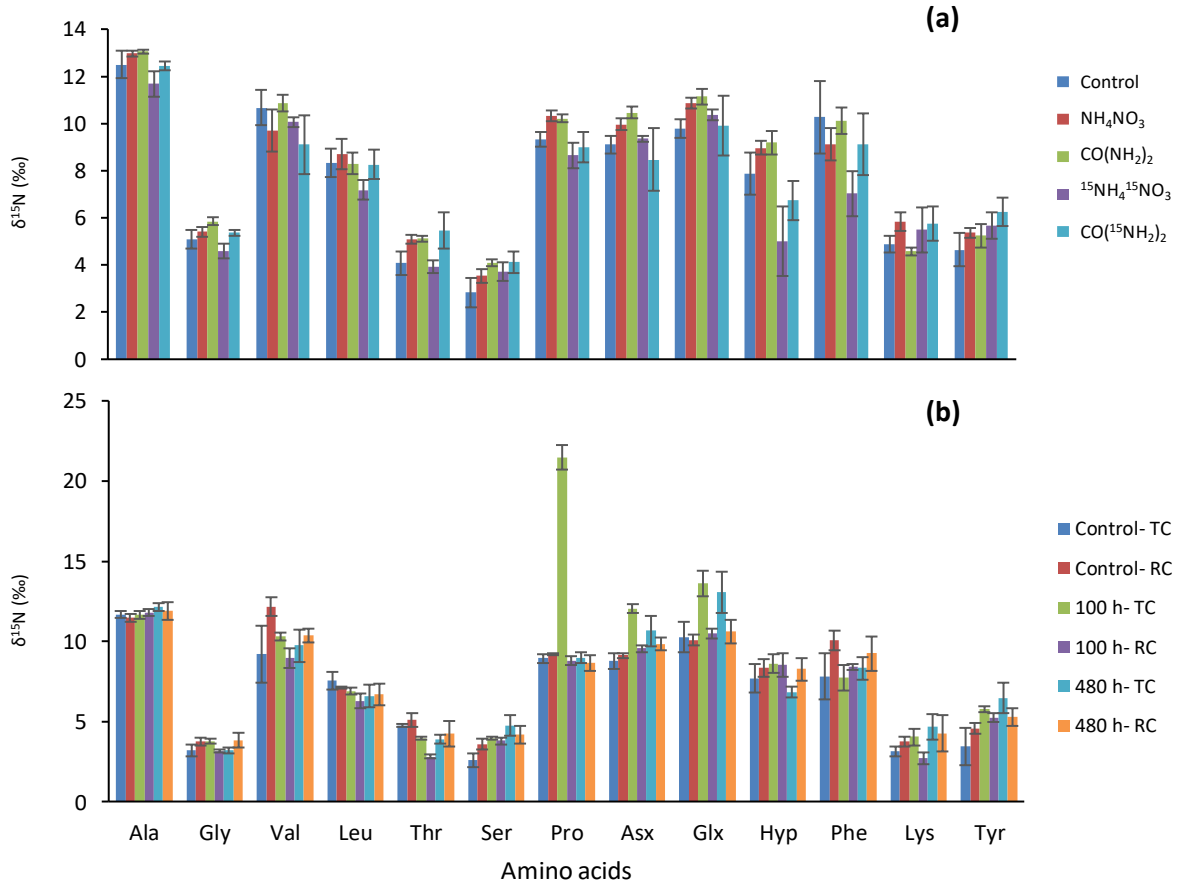


Figure 4.4. $\delta^{15}N$ values of individual hydrolysable soil AAs after application of ^{15}N-label to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*): (a) leaf-labelling technique. Leaves were either submerged in DDW for the control, natural abundance NH_4NO_3 or $CO(NH_2)_2$ or ^{15}N enriched $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ and harvested after 100 h. (b) Split-root labelling technique with DDW for the control (sampled at 100 h), or ^{15}N enriched $CO(NH_2)_2$ and sampled after 100 h or 480 h. TC- transfer compartment and RC- receiving compartment. (mean ± standard error; n= 3 or 4, outliers removed as described in Section 2.5.8)

From these results, the increase in $\delta^{15}N$ values of individual AAs can be used to determine the percentage of applied ^{15}N at time 0 incorporated into each AA harvested at the culmination of the experiment. These calculations are able to reflect the concentration and the $\delta^{15}N$ value of the AA enabling the subtle changes (increase compared to the control) in the incorporation of ^{15}N into the AAs, which cannot be seen in the figures alone (Figure 4.4), to be identified. However, over-time ^{15}N is incorporated into the plant-soil system, taken up by plants or lost out of the system and may skew results as less ^{15}N is available to be incorporated. Furthermore, in these experiments the applied ^{15}N-label was not applied directly to the soil but through the plant system. Therefore, the percentage incorporations at time t based on the moles of applied ^{15}N retained (above the control values) based on bulk ^{15}N values have also been calculated in order to help make comparisons between different experimental periods, this is known as the percentage retained. The latter calculation also enables understanding of how much of the ^{15}N present in the bulk soil is within the AAs. However, due to the small difference between the bulk $\delta^{15}N$ values and the AAs, the values for the amount of ^{15}N retained are dramatically greater than the amount incorporated from the applied ^{15}N-label. These results would suggest that the bulk soil $\delta^{15}N$ values result mostly from incorporation into AAs. This also highlights that some assimilation into AAs is taking place within the soils.

Unsurprisingly, results show very little incorporation into AAs from the applied ^{15}N-label (Table 4.10) for both the leaf-labelling and split-root labelling experiments, with Glx generally having the highest percentage incorporation. In the leaf-labelling study, $CO(^{15}NH_2)_2$ is shown to be incorporated into a greater number of AAs than $^{15}NH_4{}^{15}NO_3$, which is in agreement with previous findings showing that $CO(^{15}NH_2)_2$ results in greater ^{15}N enrichment of plant parts, which would generally mean that more ^{15}N is available to be transferred to the soil.

Table 4.10. Incorporation of the applied ^{15}N-label and retained ^{15}N in the bulk soil incorporated into individual AAs (%) for the leaf-labelling technique where leaves were submerged in $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ and harvested after 100 h, and the split-root labelling technique with $CO(^{15}NH_2)_2$ and sampled in the TC after 100 h or 480 h.

	Leaf-labelling				Split-root labelling			
	% incorporated	% retained	% incorporated	% retained	% incorporated	% retained	% incorporated	% retained
	$^{15}NH_4{}^{15}NO_3$		$CO(^{15}NH_2)_2$		100 h		480 h	
Alanine	-	-	-	0.59	0.00017	-	0.00074	35.90
Glycine	-	-	0.00055	26.62	0.00057	8.09	-	-
Valine	-	-	-	-	-	-	-	-
Leucine	-	-	-	-	-	-	-	-
Threonine	-	-	0.00098	45.33	-	-	-	-
Serine	0.00068	53.28	0.00107	44.36	0.00046	4.97	0.00098	45.18
Proline	-	-	-	-	0.00933	104.35	0.00016	2.45
Aspartic acid	0.00029	37.73	0.00047	9.84	0.00305	35.15	0.00160	61.32
Glutamic acid	0.00291	303.36	0.00289	93.22	0.00435	46.40	0.00398	243.08
Hydroxyproline	-	-	-	-	-	-	-	-
Phenylalanine	0.00033	36.07	0.00061	21.17	-	-	-	-
Lysine	-	10.42	0.00038	12.81	0.00149	13.76	0.00252	174.16
Tyrosine	0.00055	55.91	0.00061	23.62	0.00016	1.88	0.00023	13.00

4.4.5. Reverse transfer- nitrogen flow from ryegrass-to-clover

Results in this section address the objective (vii) set out in section 3.2. Application of $CO(^{15}NH_2)_2$ to ryegrass through a split-root labelling technique (Figure 4.5), results in ^{15}N enrichment throughout the whole plant system, from the soil in the TC right through to the soil in the RC. The amount of ^{15}N incorporated into the ryegrass roots in the LC is similar to the results for the LC with clover roots at 100 h (Figure 4.2) [~53300‰ (16.6 atom %) and ~49100‰ (15.6 atom %), respectively, $P>0.05$]. However, in the reverse transfer experiment, greater ^{15}N enrichment is seen in the ryegrass roots in the TC than with the clover roots at 100 and 480 h sampling points (~4130‰, 1720‰ and 910‰, respectively, although no significant difference was found between the samples). This suggests better N portioning and transfer within the ryegrass compared to clover, maybe due to ryegrass being unable to fix N_2 therefore having to adopt better strategies for N transport. However, results suggest that the ryegrass also released greater amounts of N, resulting in greater amounts of ^{15}N being available for transfer throughout the rest of the plant-soil system. The $\delta^{15}N$ values for all samples which received a $CO(^{15}NH_2)_2$ application were significantly higher than the controls. This finding is different to previous studies, using a leaf-labelling technique, for example, Rasmussen et al. (2007) found ^{15}N allocation to the roots to be higher in clover than ryegrass. The results for ryegrass-to-clover transfer are shown to have more variation than when studying clover-to-ryegrass transfer, with no clear outliers being identified. This variation can be seen in the error bars in Figure 4.5, and also in the plant biomass produced (Table 4.11). No difference was observed between the resultant biomass and N content of controls and plants labelled with $CO(^{15}NH_2)_2$.

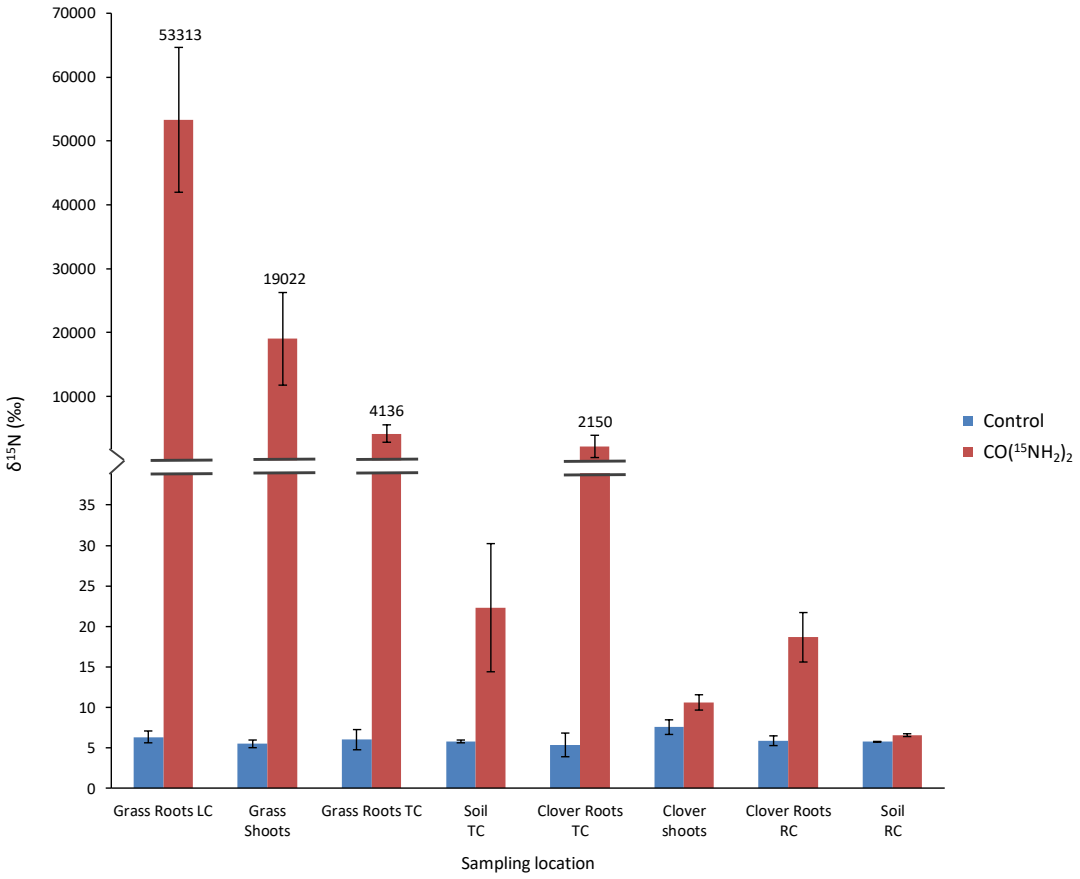


Figure 4.5. $\delta^{15}N$ values of different plant parts after application of the split-root labelling technique to ryegrass (*Lolium perenne*) and determining uptake in white clover (*Trifolium repens*). Ryegrass plants were either labelled with DDW for the control or $CO(^{15}NH_2)_2$ and sampled after 100h. LC- labelling compartment, TC- transfer compartment and RC- receiving compartment. (mean ± standard error; n= 3 or 4)

Furthermore, the reverse experiment shows greater ^{15}N enrichment of the TC soil (22.3 ± 7.9‰) than the previous two studies conducted with clover ^{15}N-labelled through leaf-labelling (6.3‰ for both $^{15}NH_4{}^{15}NO_3$ and $CO(^{15}NH_2)_2$) and split-root labelling (11.4‰ and 6.8‰ for 100 h and 480 h, respectively) techniques. The ^{15}N enrichment of the soil in the reverse experiment represents 0.35% of N-transfer from the ryegrass root into the soil N pool, this is comparable to results seen for the split-root labelling with clover at both 100 and 480 h. Furthermore, from the amount of ^{15}N applied to the ryegrass roots in the LC, results showed 0.58% incorporation into the bulk soil $\delta^{15}N$ values. The reverse experiment also showed the soil in the receiving compartment to be ^{15}N enriched compared to the control (6.6‰ and 5.8‰, respectively), this enrichment represents 0.02% of the ^{15}N-label applied into the ryegrass roots in the LC. These results show the importance of taking any N-transfer into the soil into account when calculating transfer between plants.

Table 4.11. Dry matter and N content for plant parts sampled after a 100 h split-root labelling with ryegrass roots (*Lolium perenne*) being labelled with DDW for the control or $CO(^{15}NH_2)_2$ with associated white clover (*Trifolium repens*) and sampled at 100h. LC= labelling compartment, TC= receiving compartment, and RC= receiving compartment (mean ± standard error; n= 3 or 4).). One-way ANOVA result comparing the effect of labelling substrate on the resultant plant dry matter and N content.

	Dry matter (mg plant^{-1})					
	RYEGRASS			CLOVER		
	Roots LC	Shoots	Roots TC	Roots TC	Shoots	Roots RC
Control	115 ± 80.3	220 ± 27.4	2 ± 0.8	20 ± 4.1	153 ± 20.5	22 ± 5.7
$CO(^{15}NH_2)_2$	16.48 ± 11.1	289 ± 66.5	58 ± 47.0	15 ± 5.0	114 ± 25.2	16 ± 10.2
ANOVA	NS	NS	NS	NS	NS	NS
	N content (mg plant^{-1})					
Control	1.36 ± 1.03	3.95 ± 0.37	0.03 ± 0.37	0.53 ± 0.10	5.79 ± 0.91	0.45 ± 0.20
$CO(^{15}NH_2)_2$	0.19 ± 0.12	6.48 ± 1.68	0.62 ± 0.49	0.36 ± 0.09	3.78 ± 0.80	0.44 ± 0.27
AVONA	NS	NS	NS	NS	NS	NS

NS: main effect or interaction not significant at the P<0.05 level.

Through yield-dependent expressions, the proportion of N in the legume (clover) derived from the transfer of non-legume (ryegrass) root N ($Ndft_R$) in this experiment is calculated as 1.98 ± 1.03% (calculated from Equation 2.26). This experiment exhibited moderate to high variation in both $\delta^{15}N$ values (Figure 4.5) and plant biomass (Table 4.11), which is reflected in the calculated N-transfer. Even if yield-independent expressions are used (Equation 2.28), the amount transferred is still highly variable (28.69 ± 18.83%), however, this is likely to be an over-estimate in transfer due to N-transfer into the soil not being considered. Even with the variation, the reverse transfer experiment shows greater amounts of N being transferred at 100 h than the comparison study (0.56 ± 0.18%, Table 4.8, Equation 2.26). However, larger amounts of N were seen to be transferred between clover and ryegrass at 480h (2.24 ± 0.74%) but with no comparison sampling time for the reverse N-transfer study. However, no significant difference was found between the amounts of N-transferred in either direction (P<0.05) (legume-to-non-legume or vice-versa). These results suggest significant amounts of N can be transferred in both directions (bi-directional flow), however, it is difficult to directly compare the experiments as they were undertaken at slightly different times with the conditions in the greenhouse being shown to vary, plus varying initial growth periods. For the bidirectional flow to be fully quantified and compared, these experiments should be carried out simultaneously. Despite this, it is interesting to see that in the reverse transfer experiment higher levels of ^{15}N enrichment were reached across all plant and soil samples. It is further interesting to compare

the results for bi-directional transfer, as white clover is often found to donate most N to neighbouring plant species compared to other forage legumes (Pirhofer-Walzl et al., 2012; Rasmussen et al., 2012), making these results surprising.

The amount of N-transferred between ryegrass and clover has been found to depend upon several interlinking factors: the amount of dry matter accumulated in both species, the allocation of C within the receiving species and the soil root turnover rate. Transfer from clover-to-ryegrass is greatest when there is high C investment in ryegrass root tissues and the reverse is true for ryegrass-to-clover transfer (greatest transfer when clover has a low C investment in root tissues) (Rasmussen et al., 2007). This does not explain the results from this experiment, which showed similar C contents in the ryegrass and clover roots (35.0% and 35.2%, respectively). Previous studies have also found that large amounts of N are transferred from clover-to-ryegrass when ryegrass growth is greatest (Høgh-Jensen and Schjoerring, 2000), however, all experiments conducted showed more biomass produced by the ryegrass than the clover (Table 4.5, 4.6, 4.11), although rate of growth was not measured. Furthermore, transfer of N from legumes to ryegrass is more likely when plants are grown in close association with a high legume:ryegrass ratio (Brophy et al., 1987). These experiments had roots within direct contact, but only one plant of each species, therefore, it is possible that the 1:1 ratio had a significant impact on the amount of transfer seen.

For the soil AAs in the reverse transfer experiment, the different soil compartments were found to have different concentration and distribution of soil AAs (Figure 4.6). The RC (for the control and ^{15}N applications) which solely had clover roots is shown to have a higher concentration of AAs than the TC which had both clover and ryegrass roots. This pattern is consistent for the majority of AAs with only Pro and Lys in the TC for $CO(^{15}NH_2)_2$ being greater than in the RC. This means that either the clover roots in the RC released large quantities of AAs into the soil, or more likely, there was more demand, caused by the competition of ryegrass and clover roots, in the TC for nutrients thereby depleting the AAs in the soil. However, when looking at the total hydrolysable AAs (Table 4.12), no difference was found between the control and enriched samples. Similarly, no difference in the total N and C content was found between the control and enriched samples (Table 4.12).

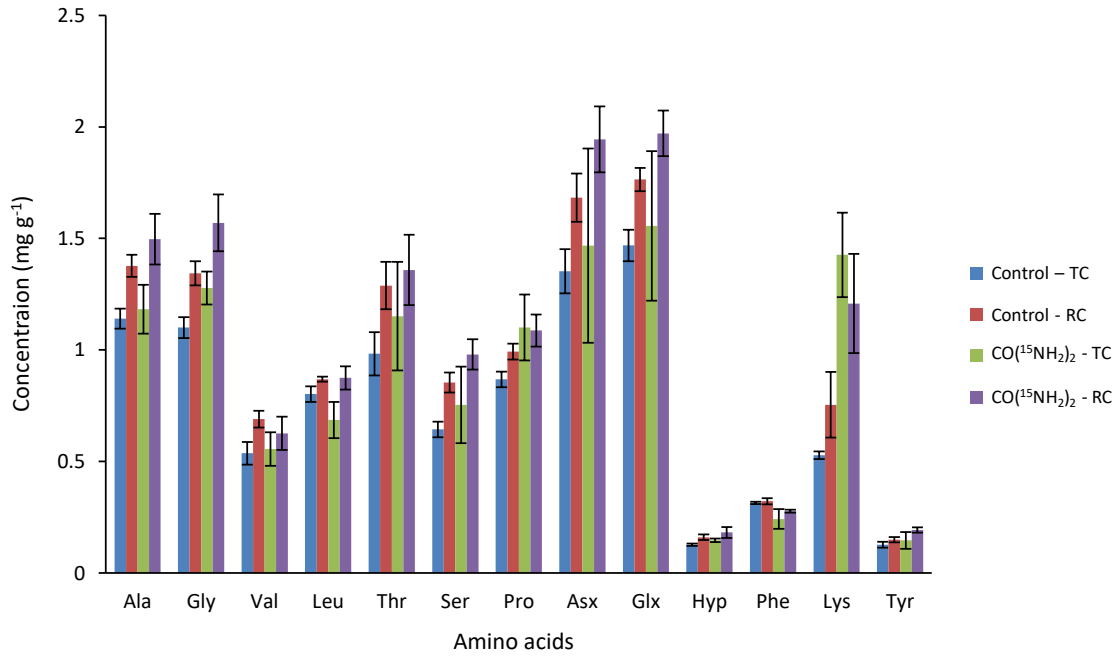


Figure 4.6. Concentration of AAs (mg of AA per gram of sample (mg g^{-1}))) in soil after application of DDW for the control or ^{15}N enriched $CO(NH_2)_2$ to ryegrass (*Lolium perenne*) via a split-root labelling technique with associated white clover (*Trifolium repens*) sampled after 100 h. TC- transfer compartment and RC- receiving compartment. (mean ± standard error; n= 4)

Table 4.12. Mean soil total N (% TN), soil total C (%TC), total soil hydrolysable AA content and total soil hydrolysable content which is N (mg g^{-1}) for the reverse transfer labelling study. LC- labelling compartment, TC- transfer compartment and RC- receiving compartment. One-way ANOVA result comparing the %TN, %TC and total hydrolysable amino acid content in the soil following the reverse transfer labelling study.

	% TN	% TC	Total hydrolysable amino acid (mg g^{-1})	Total hydrolysable amino acid N (mg g^{-1})
Control - TC	0.493	4.80	9.99	1.30
Control - RC	0.498	4.80	12.25	1.60
$CO(^{15}NH_2)_2$ - TC	0.479	4.75	11.69	1.58
$CO(^{15}NH_2)_2$ - RC	0.487	4.71	13.77	1.84
AVONA	NS	NS	NS	-

NS: main effect or interaction not significant at the P<0.05 level.

The bulk soil $\delta^{15}N$ values (Figure 4.5) are paralleled within the individual AA $\delta^{15}N$ values (Figure 4.7), with there being a relatively high amount of ^{15}N incorporation into the AAs in the TC (compared to previous studies) and a slight increase in $\delta^{15}N$ values in the RC with $CO(^{15}NH_2)_2$. This result is also paralleled when looking at the percentage incorporations into individual AAs of the applied ^{15}N-label (Table 4.13), where the label is shown to be incorporated into all AAs in the TC. Furthermore, the reverse transfer split-root study shows incorporations into the AAs between one and two orders of magnitude higher than the comparison study (Table 4.10). Similarly, to the previous study looking at transfer between clover and ryegrass (Figure 4.4b), Glx is shown to have the highest ^{15}N incorporation, followed by Asx, showing the same processes of assimilation. However, due to greater ^{15}N enrichment of the bulk soil, generally incorporation of ^{15}N into all AAs can be seen, hence the need to achieve high ^{15}N enrichment values in order to understand the processes taking place within the soil.

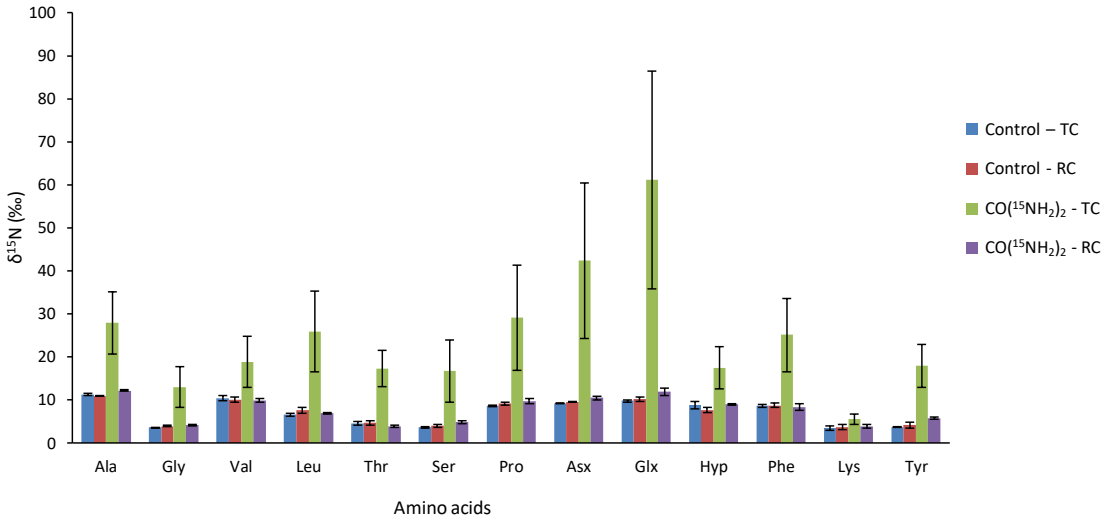


Figure 4.7. $\delta^{15}N$ values of individual hydrolysable soil AAs after application of DDW for the control or ^{15}N enriched $CO(NH_2)_2$ to ryegrass (*Lolium perenne*) via a split-root labelling technique with assocaited white clover (*Trifolium repens*) sampled after 100 h. TC- transfer compartment and RC- receiving compartment. (mean ± standard error; n= 4).

Table 4.13. Incorporation of the applied ^{15}N-label and retained ^{15}N in the bulk soil incorporated into individual AAs (%) for the reverse transfer study, where ^{15}N enriched $CO(NH_2)_2$ was applied to ryegrass (*Lolium perenne*) via a split-root labelling technique with associated white clover (*Trifolium repens*) and sampled after 100 h. TC- transfer compartment and RC- receiving compartment.

	% incorporated	% retained	% incorporated	% retained
	TC		RC	
Alanine	0.0239	78.86	0.0011	82.18
Glycine	0.0176	48.48	0.0010	70.85
Valine	0.0055	9.75	-	-
Leucine	0.0115	32.65	0.0003	15.68
Threonine	0.0147	55.33	-	-
Serine	0.0128	37.96	0.0008	67.23
Proline	0.0180	44.43	0.0004	31.84
Aspartic acid	0.0499	115.09	0.0010	77.66
Glutamic acid	0.0682	151.38	0.0014	220.49
Hydroxyproline	0.0011	3.10	-	-
Phenylalanine	0.0033	11.06	-	-
Lysine	0.0042	-	0.0008	98.98
Tyrosine	0.0015	3.14	0.0002	16.67

4.4.6. Exudation of amino acids from clover and ryegrass

Results in this section address the objective (viii) set out in section 3.2, where the quantity and pattern of AA exudation is shown to vary not only between plant species but also with time (Figure 4.8). The largest quantity of exudate is shown to be produced by clover roots at 100 h, however, this is shown to substantially reduce at 480 h (total exudates of 0.19 mg plant^{-1} reducing to 0.08 mg plant^{-1}, respectively) (Table 4.14). This decrease was found to be significant ($F_{1,6}$= 9.536, P=0.021). The clover plants in this experiment overall were seen to produce a small quantity of exudates (especially compared to previous exudate collection Figure 3.19a). The small quantity of exudates is not surprising due to the small quantity of growth media provided in this experiment, so it is likely that plants took up exudates released to support their future growth despite nutrient solution being provided, although it is unclear why this is reduced compared to the previous experiment. Furthermore, for clover there is a slight variation in exudation seen over-time. Glx is shown to be in the greatest concentration followed by Pro, then Asx at 100 h. At 480 h a slight shift occurs in exudation with Glx being in the AA in the greatest concentration, followed by Asx then Hyp with the remaining AAs having a similar concentration (all but Ala and Phe between 0.008 to 0.004 mg plant^{-1}). However, this change is quite subtle, with relatively large error bars associated with different

AA concentrations and could be within the error of the experiment, as collecting and quantifying exudates is inherently difficult. Although previous studies have shown that the composition of exudates changes with plant age (Rovira, 1956, 1969; Paynel et al., 2001a) which could explain some of the differences, also making it complex to compare between experiments when slightly different initial growth periods have been used, for example, comparing the exudates collected in Chapter 3 (Figure 3.19a). Despite this, these results are in line with previous findings where $CO(^{15}NH_2)_2$ has been applied to clover, revealing that Glx is the AA present in the greatest concentrations.

Ryegrass is shown to produce far less exudate than clover, both in terms of individual AAs and total hydrolysable content (Figure 4.8 and Table 4.14). The total hydrolysable AA content for ryegrass is observed to be significantly lower than clover at 100 h ($F_{2,9}$= 10.346, P=0.005), although no difference was found between ryegrass and clover at 480 h. Other studies have found the efflux of AAs from legumes to be greater than that from grasses (Lesuffleur et al., 2007), although it is interesting to see that this only holds true for clover sampled at 100 h. This could be as a result of a change in the nutrient status of the plant, where nutrient stress was greater at 480 h resulting in the re-uptake of AAs, as plant exudation is found to compose of a net release of compounds from both the influx and efflux (Jones and Darrah, 1994).

For ryegrass, Glx is shown to be present in the greatest concentration, followed by Asx. Again, Figure 4.8 suggests a different pattern of exudation than previous studies, which have shown that Gly and Ser are by far the major AAs exuded from plants (Paynel et al., 2001a). Again, suggesting that there is some correlation between the AA profiles in the roots and exudates, where Gln, Glu and Asp are the major AAs in ryegrass roots (Paynel et al., 2001a). This was discussed in terms of clover exudates in Chapter 3.

The difference in the quantity of exudates produced by ryegrass and clover is important to this study. Typically, legume exudates are found to contain more amino N than non-legumes (Hale et al., 1978). Comparable bi-directional N-transfer was seen in this experiment, however, this further questions the processes involved in transfer from ryegrass-to-clover, suggesting that other major compounds are released by ryegrass and are responsible for N-transfer, such as inorganic N. This further highlights that assimilation into AAs must have occurred to result in the increase in AA $\delta^{15}N$ values (Figure 4.8).

Due to the low concentration of AAs collected in this experiment, it was not possible to quantify Lys or Tyr, furthermore, the low concentration made it impossible to collect any isotope data on the routing of ^{15}N into the individual AAs, this has been a problem with other studies, such as Hertenberger and Wanek (2004). Additionally, no control exudates were quantified due to problems with the methodology. Due to the nature of the samples and the fact that the samples collected were of low mass, all of the exudate collected had to be used in one AA hydrolysis and derivatisation process, meaning the sample could not be repeated, without the whole experiment being re-run.

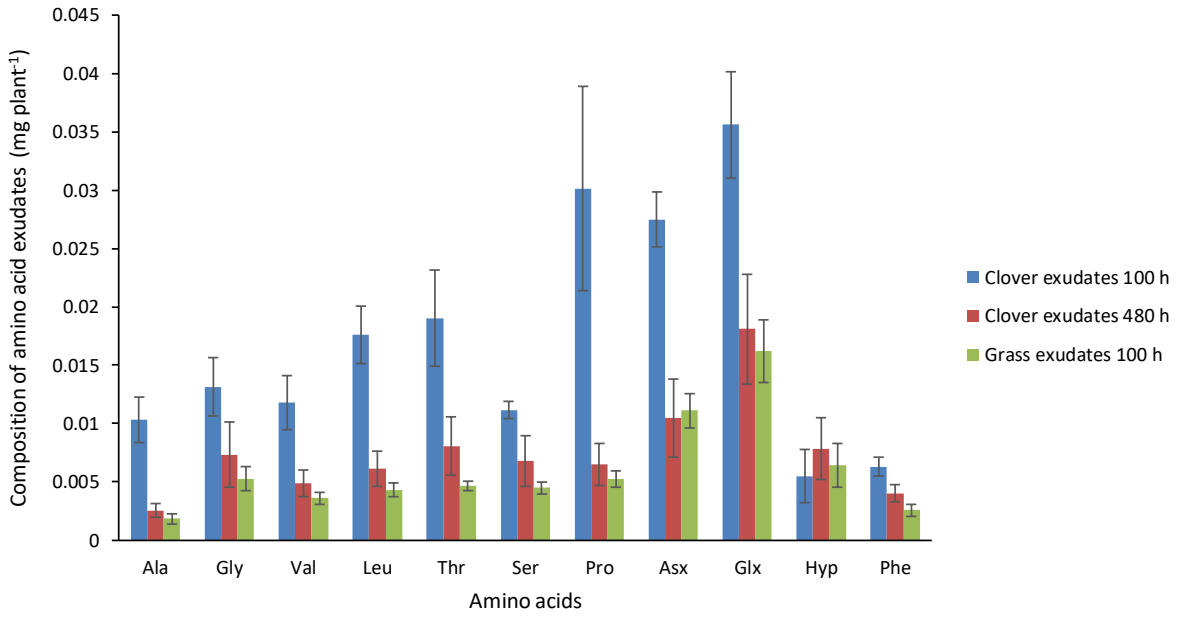


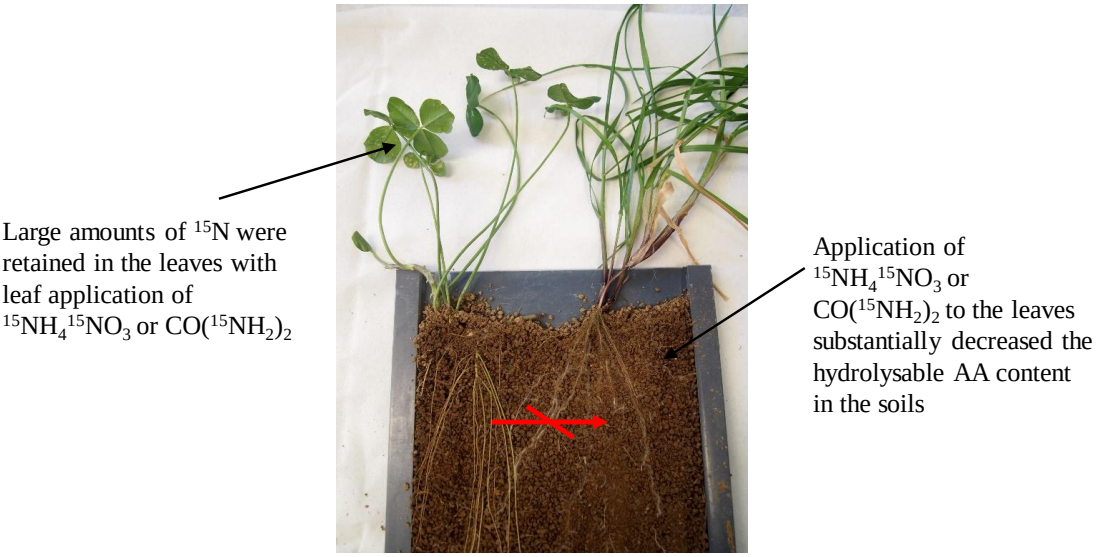
Figure 4.8. Composition of AAs recovered from sand in the LC [mg of AA produced by each plant in each incubation tube over the experimental period (mg plant^{-1})] following the application of ^{15}N enriched $CO(NH_2)_2$ to roots of white clover (*Trifolium repens*) and ryegrass (*Lolium perenne*) grown using a split-root labelling technique. Plants were sampled and exudates were collected after 100 h and 480 h for clover, and 100 h only for ryegrass. Concentrations of exudates have been corrected to account for recovery rates of individual AAs (Figure 3.15). (mean ± standard error; n=4).

Table 4.14. Total soil hydrolysable AA content and total hydrolysable content which is N (mg plant^{-1}) recovered from sand in the LC following the application of ^{15}N enriched $CO(NH_2)_2$ to roots of white clover (*Trifolium repens*) and ryegrass (*Lolium perenne*) grown using a split-root labelling technique. Plants were sampled and exudates were collected after 100 h and 480 h for clover, and 100 h only for ryegrass. One-way ANOVA result comparing clover exudates at 100 h and 480 h, and clover and ryegrass exudates at 100 h.

	Total hydrolysable amino acid (mg plant^{-1})	Total hydrolysable amino acid N (mg plant^{-1})
Clover exudates 100 h	0.188	0.022
Clover exudates 480 h	0.083	0.010
Ryegrass exudates 100 h	0.066	0.008
ANOVA interaction		
Clover 100 h * Clover 480 h	P=0.021	-
Clover 100 h * Ryegrass 100h	P=0.005	-

4.4.7. Summary of findings within Chapter 4

Leaf-labelling in rhizotrons



Split-root labelling in incubation tubes

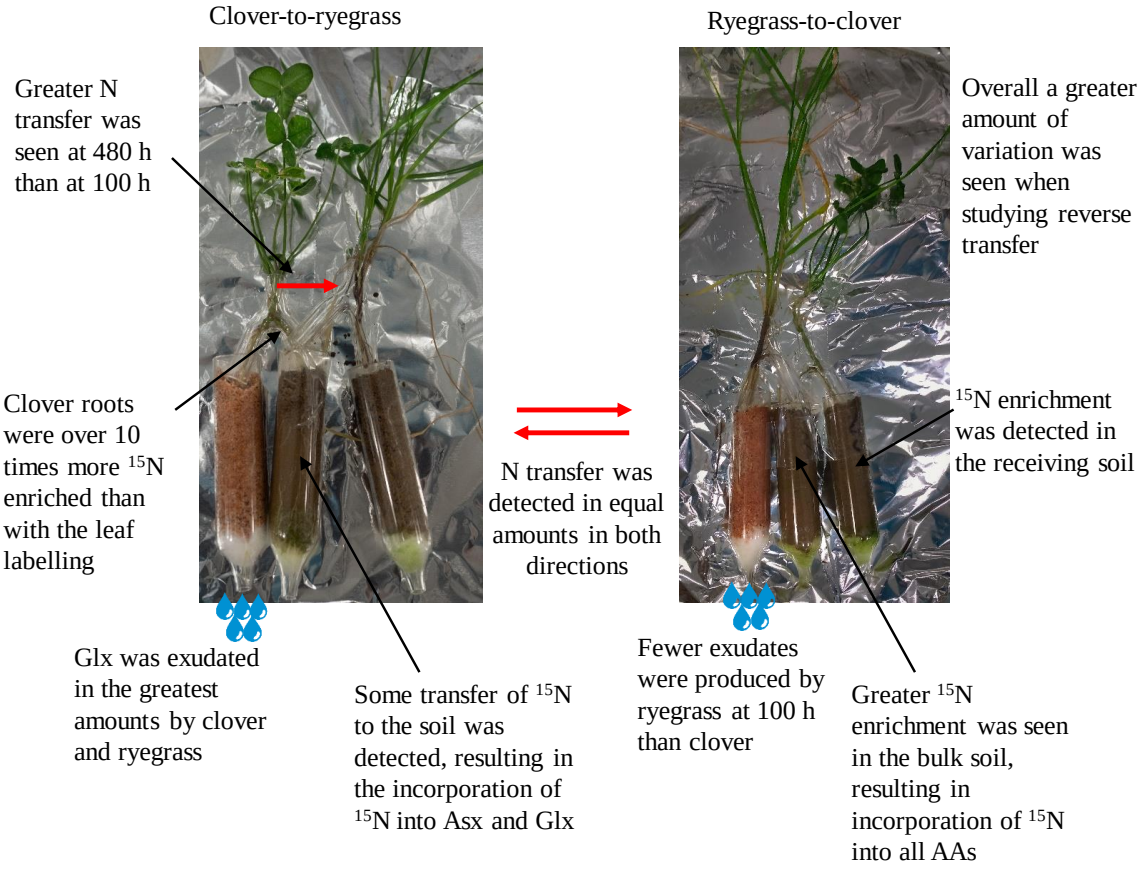


Figure 4.9. Summary figure of experiments conducted within this chapter, which studied the use of the leaf-labelling technique in rhizotrons and the split-root labelling technique in incubation tubes to study N-transfer from clover-to-ryegrass and vice-versa.

4.5. Conclusion

The methods presented in Chapter 3 have been further developed in this chapter to look at transfer between two plant species: clover and ryegrass. The findings presented in this chapter will further assist in the development of a robust method used in later chapters. Originally the leaf-labelling technique was proposed as the best-method for introducing ^{15}N enriched compounds to plants (Chapter 3) due to its ease of application and field applicability. Despite this and the fact that the shoot-labelling technique is the most commonly used method to measure direct N-transfer between plants (Chalk et al., 2004), this study has raised concerns over its application, especially on plant physiology.

Important specific findings, relating to the objectives set out in section 4.2, include:

(i) Very low ^{15}N enrichment using the leaf-labelling technique compared to split-root labelling, resulting in negligible N-transfer between plants being seen, which is a fundamental part of this work.

(ii) Comparison of unlabelled and labelled substrates using the leaf-labelling technique were not found to alter plant biomass or plant tissue C and N contents.

(iii) The different methods for calculating N-transfer were compared, from this the most appropriate calculation for this work is Equation 2.26, taking into account the ^{15}N enrichment of the soil and is yield-dependent.

(iv) In-depth study into the hydrolysable AA content of soils with labelled and unlabelled substrates, revealed that ^{15}N enrichment, using the leaf-labelling technique, substantially decreases the concentration of soil AAs, which is not seen through split-root labelling. Although no effect on the total soil C and N content was seen. Applying N containing compounds to the leaves does not follow the natural pathway for assimilation of N, which is likely to have been responsible for the observation made.

(v) Some incorporation of ^{15}N into individual soil AAs was found for the split-root labelling technique studying clover-to-ryegrass transfer, namely Glx and Asx, suggesting some assimilation by microbial biomass. However, greater overall enrichment is needed to fully probe processes involved in N-transfer. Whereas, the leaf-labelling technique showed no incorporation of ^{15}N into soil AAs.

(vi) Determining the percentage of applied ^{15}N incorporated into the total hydrolysable AA pool is shown to be a useful tool for identifying subtle changes which $\delta^{15}N$ values alone do not capture. The results unsurprisingly showed little incorporation with the leaf-

labelling and split-root labelling techniques, with Glx generally having the highest percentage incorporation.

(vii) Reverse N-transfer was found in the ryegrass-to-clover experiment. Overall greater ^{15}N enrichment was achieved for the study on N-transfer from ryegrass-to-clover, than clover-to-ryegrass, enabling the transfer of ^{15}N to be seen across the whole spectrum of AAs. However, experiments would need to be run simultaneously to fully calculate net transfer between plants and how this varies in a field environment is questioned.

(viii) Ryegrass is shown to produce far fewer AA exudates than clover at 100 h, with Glx being exuded in the greatest amounts for both species.

The major implications from this chapter reveal that: bi-directional flow of N between clover and ryegrass is shown to exist, with substantial transfer in both directions and in terms of a robust method for future chapters the split-root labelling technique is preferred over that of the leaf-labelling technique. The split-root labelling technique can be easily manipulated in later chapters to investigate a range of different treatments and their effect on N-transfer. The split-root labelling technique could be used for a wide range of different studies such as to determine the fate of different compounds and rate of uptake (e.g. fertilisers) in order to achieve optimum N-transfer.

Chapter 5

Investigation of the role of exudation and decomposition in nitrogen transfer from clover-to-ryegrass

5. Investigation of the role of exudation and decomposition in nitrogen transfer from clover-to-ryegrass

5.1. Introduction

Nitrogen is often the nutrient limiting crop growth and sustaining high yields, which results in conventional agricultural practices using synthetic fertilisers to address supply. Alternatively, N-fixing legumes, such as clover, alfalfa or soybean can be used to improve N supply to an associated crop (Thilakarathna et al., 2016). However, legume and non-legume cropping systems have been limited through a lack of understanding of the N-transfer pathway. N-transfer is the process and movement of N through deposition by one plant ("N donor"), followed by uptake by a neighbouring plant ("N receiver") (Jensen, 1996b). To maximise the benefits of the association, a better understanding of the underlying mechanisms and factors which govern N-transfer are needed (Frey and Schüepp, 1992; Thilakarathna et al., 2016). Typically, three major N-transfer pathways from legumes to non-legumes are classified:

(i) Death, decay and decomposition, followed by mineralization of root and nodular tissue, which can result from senescence or sloughing-off of root epidermal cells (Brophy and Heichel, 1989; Murray and Clements, 1998).

(ii) Active or passive release (plant exudation) of nitrogenous compounds, including AAs by the legume root and nodules (Virtanen et al., 1937; Ta et al., 1986; Wacquant et al., 1989).

(iii) Mycorrhizal associations between the plants, either directly through common mycorrhizal networks (CMNs) or by indirect mycorrhizal mediated transfer (Bethlenfalvay et al., 1991).

These three major routes of N-transfer are usually discussed in terms of belowground transfer, although aboveground mechanisms do exist, such as: shoot litter decomposition and animal consumption of foliage and return as excreta (Figure 5.1) (Ledgard, 1991, 2001; Dahlin and Stenberg, 2010; Rouquette and Smith, 2010; Peoples et al., 2015). Furthermore, the roles of shoot, root and nodules, herbivores and pathogens are often not considered, these have been suggested as playing a role within rapid N-transfer, through releasing cell contents. Although there is no scientific consensus whether their role plays a major part within N cycling (Brophy and Hiechel, 1989; Murray and Clements, 1992, 1994; Hatch and Murray, 1994; Murray et al., 1995; Murray and Clements, 1998).

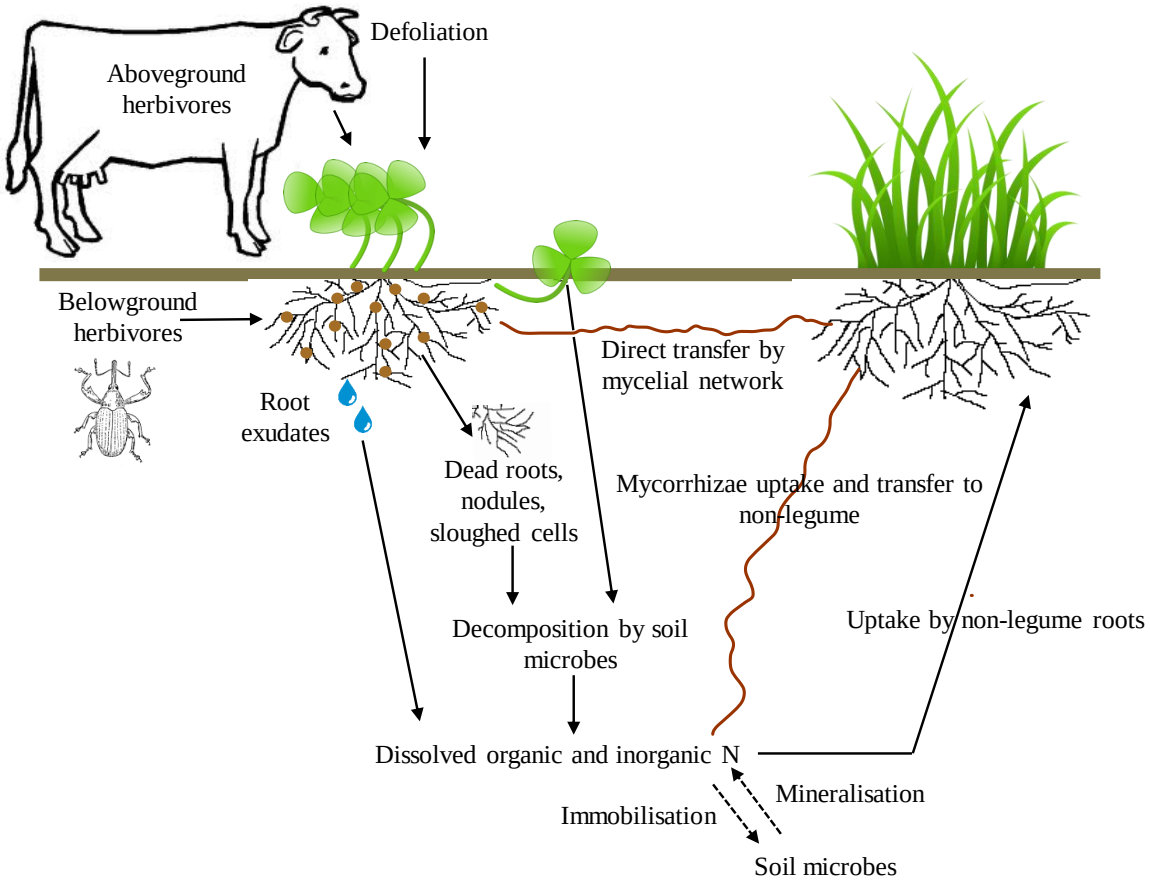


Figure 5.1. Possible aboveground and belowground N-transfer mechanisms from legume to non-legumes.

The majority of these N-transfer pathways can be described in terms of being "direct" or "indirect", i.e. N-transfer between plants without mineralisation, or through mineralisation followed by plant uptake (Thilakarathna et al., 2016). Decomposition is often considered the most indirect pathway, due to material firstly needing to be decomposed, then mineralised before being available for plant uptake (Fustec et al., 2010). Although all compounds present in the soil are subjected to microbial mineralisation, for example, plant exudate can be part of both a direct and an indirect pathway, as they can be rapidly mineralised by microbes due to their low C:N ratio (Paynel et al., 2001a, 2008; Jalonen et al., 2009a,b; van Kessel et al., 2009; Uselman et al., 2010). It has previously been suggested that direct N-transfer from a legume to a non-legume might not occur in all soil conditions or alternatively it may only occur gradually (Peoples and Craswell, 1992).

There is much conflicting evidence of the relative importance of each N-transfer pathway and this has been the subject of many discussions. Several studies comparing the different N-transfer pathways have considered nodule and root decomposition to be more crucial in N-transfer than root exudates or mycorrhizae fungi mediated pathways (Ta and Faris, 1987; Trannin et al., 2000; Sierra et al., 2007). Although there is increasing evidence for the role of interplant N-transfer through CMNs in mixed cropping systems and it is considered to be the most direct route for N-transfer (Stern, 1993; Johansen and Jensen, 1996; Thilakarathna et al., 2016; and see references within Chapter 6). However, others have concluded that N-transfer mostly occurs through exudation, not mycelial networks (Jalonen et al., 2009a). Nevertheless, decomposition is a major part of the N-cycle, due to the fact that all living organisms possess and are essentially made up of large amounts of assimilated N. In terrestrial ecosystems, the primary source of mineral N, for biological activity, is decomposition (Parton et al., 2007), therefore, the rate of decomposition is fundamental to plant N uptake.

Defining the mechanisms which affect N-transfer is complex, due to the number of biotic and abiotic factors which also interact with below and aboveground cycling of N. Abiotic factors, include: irrigation, temperature, light, soil available N, N fertiliser application and overall growing conditions. While biotic factors which affect N-transfer, include: plant species (including cultivar), genetic variability, root contact (inter alia species proximity), plant density, growth stage, production year, defoliation, plant death, plant herbivores and land management practices (Wacquant et al., 1989; Fujita et al., 1992; Murray and Clements, 1998; Chalk et al., 2014; Thilakarathna et al., 2016). Many of the abiotic factors which are at play in natural environments are beyond the control of the growers, whereas biotic factors can be more easily manipulated to increase N-transfer between plants (Thilakarathna et al., 2016). Further to this, the importance of each N-transfer pathway has been found to vary between legume species, therefore, more information is needed on the processes and efficiency of N-transfer between plants to define the best combination for yield maximisation (Ta and Faris, 1987).

5.2. Objectives

The work presented in this chapter uses the methods developed in Chapters 3 and 4, which looked at ways of introducing a ^{15}N-label into a clover plant and estimating the transfer of N from a legume to the associated non-legume species. This chapter will look at some of the main processes involved in N-transfer from clover-to-ryegrass, including the belowground process of exudation and root decomposition compared to the aboveground process of shoot decomposition. The role of soil biota (mycorrhizal and root herbivory) in N-transfer will be examined later in Chapter 6. This chapter will examine these different processes and their relative contribution to N-transfer to determine whether they can be maximised in an intercropping system in order to help develop land management strategies. This is centred around the fact that in natural ecosystems the processes which are involved in conserving productivity and stability could be combined within agricultural land-use management practices to help develop more sustainable agricultural systems (Wahbi et al., 2016).

This chapter will investigate one of the central hypotheses of this thesis (H1). It is hypothesised that decomposition will play a larger role in ^{15}N uptake in ryegrass and therefore N-transfer from clover-to-ryegrass than does exudation. Removing the clover shoots by cutting will accelerate death and decomposition and substantially increase the ^{15}N uptake in ryegrass and therefore N-transfer from clover-to-ryegrass.

The specific objectives of this work are to:

(i) Compare uptake of ^{15}N in clover and ryegrass using the split-root labelling technique and applying different treatments modifying the N-transfer pathways from clover-to-ryegrass.

(ii) Determine whether modifying the different N-transfer pathways effects plant growth and plant C and N content in clover or ryegrass.

(iii) Compare N-transfer from clover-to-ryegrass using the split-root labelling technique and applying different treatments modifying the N-transfer pathways from clover-to-ryegrass.

(iv) Monitor the hydrolysable soil AA concentrations for response to the ^{15}N addition to clover plants and modifications to the different N-transfer pathways between plants.

(v) Determine and examine the patterns in individual hydrolysable soil AA $\delta^{15}N$ values in response to the addition of $CO(^{15}NH_2)_2$ *via* the split-root labelling technique and modifications to the different N-transfer pathways between plants.

(vi) Determine the percentages of applied ^{15}N incorporated into the total hydrolysable AA pool and whether the different N-transfer pathways between plants effects the percentage incorporation.

(vii) Comment on the development of new land-use management strategies for the sustainable transfer of N from clover-to-ryegrass.

5.3. Materials and methods

Incubation tubes were set up as described in Section 2.2.10, but with one tube of sand acting as the labelling compartment and only one additional tube as the transfer compartment. Cuttings of clover and ryegrass plants were taken and allowed to grow in pots of compost for six weeks to enable the roots to establish (growing period between April and May 2017, average temperature can be found in Section 2.2.5). Twelve sets of incubation tubes were set up where the clover roots were divided between the LC and TC, with the ryegrass being rooted in the TC only, this was done for the control treatment receiving DDW only ("control"), the treatment receiving $CO(^{15}NH_2)_2$ only ("no treatment") and the treatment receiving $CO(^{15}NH_2)_2$ where the clover leaves were later cut ("cut clover"). A further eight individual TC tubes were filled with soil where the ryegrass plant was solely rooted in them. Additionally, four sets of incubation tubes were made up with the clover roots divided between the LC and TC which were both filled with 25 g of sand ("Clover exudates"). Plants in incubation tubes were moved into the contaminant section of the greenhouse, to reduce the likelihood of whitefly infestations during the running of the experiment (which were a problem in the greenhouse at the time). Plants were left to grow for a further six weeks before the experiment commenced, temperatures for the duration of the experiment can be found in Table 5.1.

Table 5.1. Maximum, minimum and average temperatures (°C) in the containment section of the greenhouse during the experiment.

	Max	**Min**	**Avg**
May-17	29.1	14.6	18.7
June-17	30.7	15.1	19.1
July-17	29.0	15.5	20.1

To produce fresh ^{15}N-labelled clover plant material ("clover incorporated" treatment), an additional four sets of incubation tubes with the clover roots divided between the LC and TC (as in Chapter 3) were used, clover plants were allowed to establish for six weeks, before 30

mM $CO(^{15}NH_2)_2$ at 98 atom % was injected into the LC (0.25 mL x 4), clover plants were sampled after 100 h as this had previously been seen to a good compromise between substantial ^{15}N enrichment in the clover roots and shoots and labelling time (Chapters 3 and 4). The freshly produced ^{15}N enriched clover shoots were finely chopped and 0.7 g was incorporated into the TC soil with one ryegrass plant rooted at the time of ^{15}N-labelling.

The TC in this experiment also acted as the treatment compartment, with four repeats per treatment being used to study five different N-transfer pathways. After six weeks of growth within incubation tubes, the substrates were introduced into the LC by injecting (0.25 mL x 4) with DDW for the control ("Control") or 30 mM $CO(^{15}NH_2)_2$ at 98 atom % into the "no treatment", "clover cut" and "clover exudates". For the exudates treatment, at the time of labelling, one soil filled TC with ryegrass was placed underneath the sand filled TC (held by two test tube racks placed one above another) and connected with nalgene clear plastic PVC tubing (5 cm by ø 7mm). The ryegrass soil was only watered using leachates from watering with nutrient solution of the sand filled transfer compartment above (Figure 5.2). For the clover cut treatment, the clover shoots were removed at 100 hours after initial labelling, to do this the shoots were cut to the level of the Y-tube, leaving the roots connected between the LC and TC. This treatment aimed at killing the clover plant to leave the roots to decompose.

The experiment was halted 480 h after LC and TC tubes were sampled. At the end of the experiment plant leaves were immediately cut to halt any further transfer. Subsequently, each incubation tube was deconstructed and plant parts and soils separated. All samples were immediately placed in the freezer, then latterly freeze-dried and weighed. All plant and soil samples were analysed for bulk $\delta^{13}C$ and $\delta^{15}N$ value determinations (Section 2.3). In addition, AAs were also extracted from the soils, derivatised and analysed by GC-FID (quantification) and GC-C-IRMS (compound specific $\delta^{15}N$ value determination) (Section 2.4).

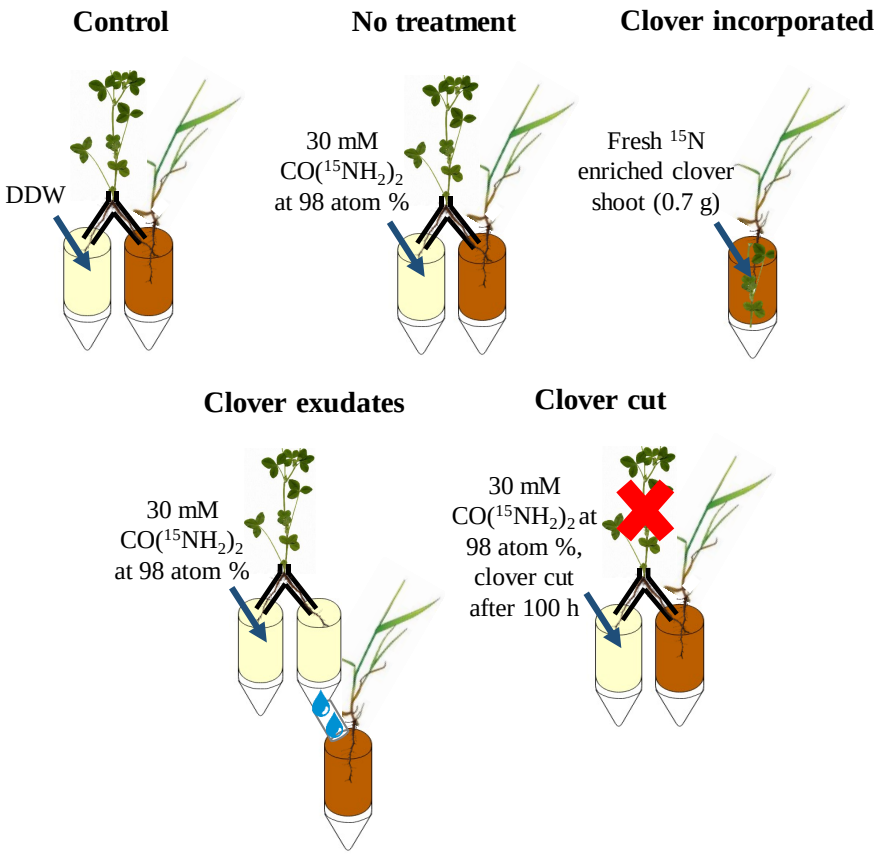


Figure 5.2. Experimental set-up used in Chapter 5 to study the N-transfer pathways between white clover (*Trifolium repens*) and ryegrass (*Lolium perenne*) using a split-root labelling technique with labelling and transfer compartments (LC and TC, respectively). Clover plants were either labelled with DDW for the control, ^{15}N enriched $CO(NH_2)_2$ only ("no treatment"), ^{15}N enriched $CO(NH_2)_2$ clover shoot incorporated into the ryegrass soil ("clover incorporated"), ^{15}N enriched $CO(NH_2)_2$ exudates feed to the ryegrass plant ("clover exudates") or ^{15}N enriched $CO(NH_2)_2$ clover plant killed by cutting after a 100 h labelling period ("clover cut").

5.4. Results

5.4.1. Effect of N pathway on partitioning of ^{15}N

Results in this section address the objectives (i) and (ii) set out in section 5.2. The split-root labelling technique was shown to be effective at substantially ^{15}N enriching all plant parts as well as allowing different treatments to be applied to the soil in the TC (Figure 5.3). The clover shoots and roots were all noticeably more ^{15}N enriched than the unlabelled control, showing that the ^{15}N-label was taken up into the plant parts successfully. No significant difference was found between clover plant parts enrichment with ^{15}N, showing that the different treatments applied did not affect plant uptake of the applied ^{15}N-label in the LC. Importantly, the lower ^{15}N enrichment of the clover shoots in the clover incorporated treatment which only had a 100

h labelling period were not significantly lower. Furthermore, very similar ^{15}N enrichment of clover was found between this study and previous studies where the method was developed (Figure 4.2), with no significant difference between the ^{15}N enrichment of any clover plant parts showing the consistency of ^{15}N uptake by clover between studies (Table 5.2).

The TC soil was shown to be ^{15}N enriched compared to the control for all treatments applied, a significant difference was found between treatments ($F_{4,15}$= 56.44, P= 0.000). The clover incorporated treatment was found to be different and higher than the other four treatments, with no significant different found between all other treatments. However, the ^{15}N enrichment of bulk soil in the clover incorporated treatment is a bias representation of the true ^{15}N enrichment of the bulk soil due to the fact that small fragments of the ^{15}N enriched clover shoots incorporated could not be removed. Therefore, comparing the remaining four treatments' $\delta^{15}N$ values of the soil, a significant difference was found between treatments ($F_{3,12}$= 7.08, P= 0.005), where the control was found to be significantly different and lower than the no treatment receiving $CO(^{15}NH_2)_2$ only, however, the other two treatments were not seen to differ from the control. Further to this, the no treatment soil was seen to be significantly more ^{15}N enriched than both the control and the clover exudates treatment. This result is not surprising, as the percentage incorporation of the applied ^{15}N-label into the TC soil was low, but highest for the no treatment (1.06%) compared to the clover exudates treatment (0.33%) and the clover cut treatment (0.60%) (Table 5.3). For an increase in ^{15}N enrichment in the soil to be achieved, ^{15}N-transfer must be from the clover roots. Results showed that the ^{15}N enrichment in the soil represented a low ^{15}N-transfer from the clover roots into the soil, with the no treatment and clover cut treatments showing a similar amount of transfer (2%) and the clover exudates treatment seeing far less (0.42%) (Table 5.3). A significant difference was found between treatments ($F_{2,9}$= 4.49, P= 0.045), where the clover exudate treatment was significantly different and lower than the cut clover and no treatment.

For this experiment, despite very similar ^{15}N enrichments being seen in the clover plant parts compared to previous experiments, the soil became noticeably more ^{15}N enriched than the previous studies (Chapter 3 and 4). For example, a comparable $CO(^{15}NH_2)_2$ treatment having an average of 6.79‰ (Figure 4.2), compared with this study of 38.43‰ (Figure 5.3), this is probably due to removing further N dilution from the RC soil. Despite this, the bulk soils for the clover exudates and clover cut were not found to be significantly more enriched than the

control soil despite greater plant ^{15}N enrichments. Furthermore, there was much greater incorporation of the applied ^{15}N-label into bulk soil in this study for all treatments than in previous comparable studies, for example, 0.01% (Table 4.7), compared to 2.08% in this study (Table 5.3).

Results also show N-transfer to the ryegrass roots and shoots, with these being more ^{15}N enriched than the control. For the ryegrass roots, the clover incorporated treatment showed the greatest ^{15}N enrichment at 1210‰ (0.8 atom %), followed by the no treatment > clover cut > clover exudates, compared to the control at 4‰. A significant difference was found between ryegrass root $\delta^{15}N$ values of different treatments ($F_{4,15}$= 9.03, P= 0.001), where the control was different and lower than the clover incorporated treatment, with no significant difference between all other treatments applying $CO(^{15}NH_2)_2$ and the control. For the ryegrass shoots, again the clover incorporated treatment showed the greatest ^{15}N enrichment at 860‰ (0.7 atom %), compared to the control at 5‰. A significant difference was found between ryegrass shoot $\delta^{15}N$ values of different treatments ($F_{4,15}$= 20.33, P= 0.000) (Table 5.2), where the control was different and lower than the clover incorporated treatment, with no significant difference between all other treatments applying $CO(^{15}NH_2)_2$ and the control. Again, if the highly enriched clover incorporated treatment ryegrass roots and shoots are excluded from the statistical analysis, a significant difference is found for the ryegrass roots between different treatments ($F_{3,12}$= 6.27, P= 0.008), where the control was different and lower than the no treatment, with no significant different between all other treatments applying $CO(^{15}NH_2)_2$ and the control. However, the $\delta^{15}N$ values of the no treatment are seen to be significantly higher than the control and the clover exudate treatment, but not the cut clover. Similarly, a significant difference was found between ryegrass shoot $\delta^{15}N$ values of different treatments ($F_{3,12}$= 11.36, P= 0.001), where the no treatment was seen to be significantly higher than all the other treatments, however, these were not found to be significantly different from each other.

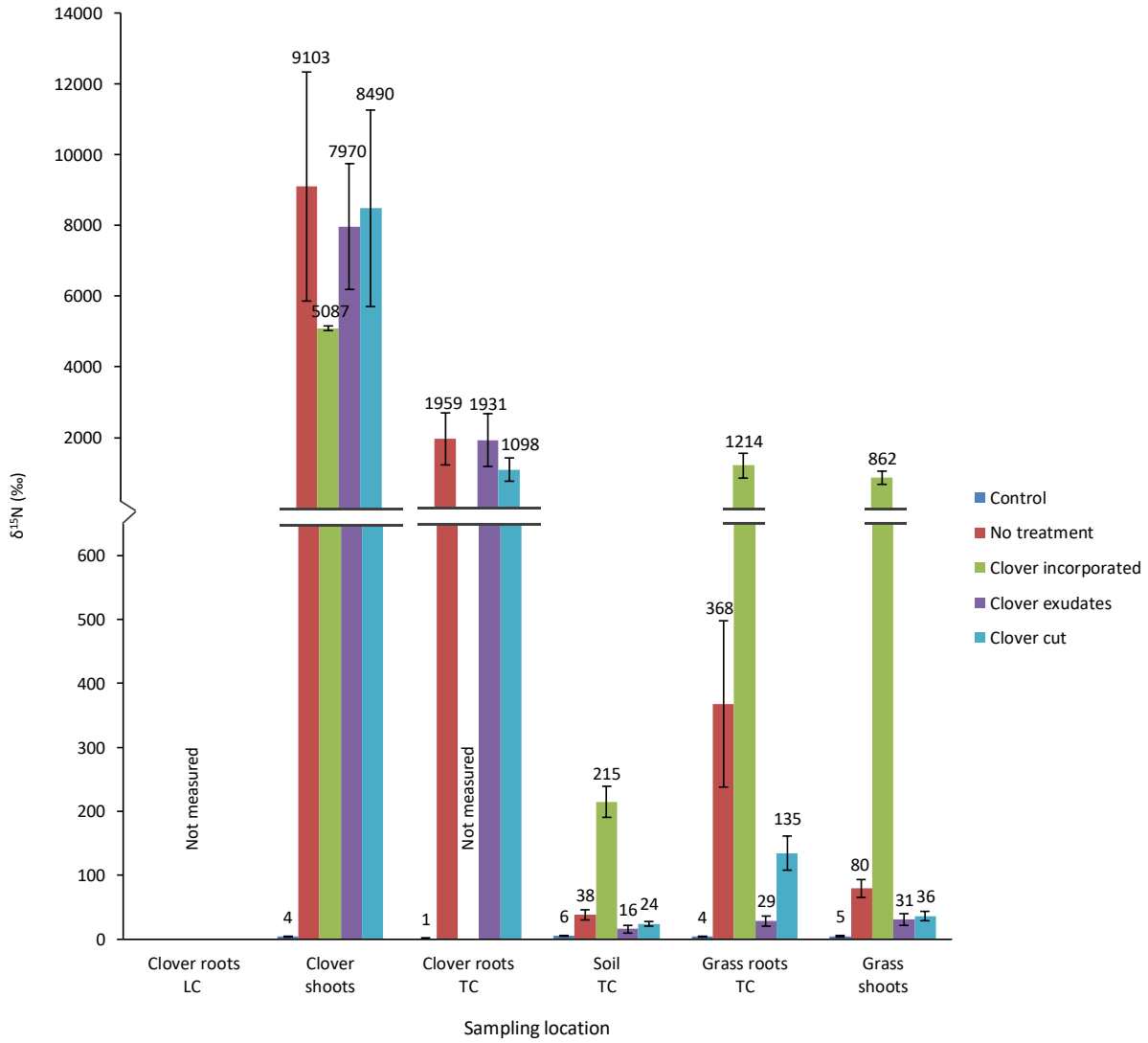


Figure 5.3. $\delta^{15}N$ values of different plant parts after application of the split-root labelling technique to white clover (*Trifolium repens*) and determining uptake in ryegrass (*Lolium perenne*). LC- labelling compartment, TC- transfer compartment. (mean ± standard error; n= 4).

Table 5.2. Statistical results for experiment looking at the split-root labelling technique to white clover (*Trifolium repens*) with different treatments to study exudation and decomposition.

ANOVA Interaction	P-value
Clover shoots: All treatments applying ^{15}N	NS
Clover roots TC: All treatments applying ^{15}N	NS
Soil TC: All treatments	P=0.000
Ryegrass roots TC: All treatments	P=0.001
Ryegrass shoots: All treatments	P=0.000

NS: main effect or interaction not significant at the P<0.05 level.

Table 5.3. Percentage transfer of the ^{15}N-label from plant derived N to TC soil [N derived from rhizodeposition (Ndfr)] and percentage incorporation of the applied ^{15}N-label into the bulk soil. One-way ANOVA result comparing the effect of different treatments on Ndfr and the incorporation of ^{15}N label into the bulk soil.

	Ndfr (%)	Incorporation of ^{15}N label into bulk soil (%)
No treatment	2.08 ± 0.58	1.06 ± 0.24
Clover incorporated	4.19 ± 0.50*	6.73 ± 0.76
Clover exudates	0.42 ± 0.09	0.33 ± 0.20
Clover cut	2.04 ± 0.50	0.60 ± 0.10
AVONA	P=0.045	P=0.005

*Calculation based on enrichment of clover shoots incorporated into the bulk soil, however, small fragments of clover shoots incorporated into the bulk soil could not be separated
NS: main effect or interaction not significant at the P<0.05 level.

A substantial amount of biomass was produced by the clover and ryegrass plants during the experiment (Table 5.4). In comparison to previous experiments, total clover biomass was much greater, for example, the control treatment produced 487 mg, compared to only 166 mg in previous experiments (Table 4.6). For ryegrass, biomass production did not vary so much between experiments, for example, the control treatment producing 455 mg, compared to 408 mg in previous experiments (Table 4.6). The control was seen to have the greatest total clover biomass and the clover exudates treatment was seen to have the greatest total ryegrass biomass. However, no difference was found between treatments and the clover and ryegrass biomass produced. The C and N content for the different plant parts was seen to be fairly consistent between different treatments, however, the N content of the ryegrass shoots was seen to be substantially higher in the clover incorporated treatment than the control (Table 5.5). However, for the majority of plant parts no difference was found between the C and N content and C:N ratio with different treatments (Table 5.5). A significant difference was found for the C:N ratio of the ryegrass shoots ($F_{4,14}$= 9.75, P= 0.001), where the clover incorporated treatment was seen to be significantly different and lower than the other treatments, which were not significantly different to each other.

Table 5.4. Dry matter (mg plant^{-1}) for plant parts sampled after a 480 h labelling period using the split-root labelling technique to white clover (*Trifolium repens*) and associated ryegrass (*Lolium perenne*). LC= labelling compartment, TC= receiving compartment (mean ± standard error; n=4). One-way ANOVA result comparing the effect of treatments on the resultant plant dry matter.

	Dry matter (mg plant^{-1})						
	CLOVER				RYEGRASS		
	Roots LC	Shoots	Roots TC	Total	Roots TC	Shoots	Total
Control	78.6 ± 14.1	312 ± 15.7	97.0 ± 28.6	487 ± 17.9	155 ± 49.9	300 ± 37.9	455 ± 78.3
No treatment	88.4 ± 13.1	231 ± 38.3	57.3 ± 17.6	377 ± 53.0	455 ± 114	434 ± 54.3	889 ± 160
Clover incorporated	n/a	n/a	n/a	n/a	252 ± 30.6	457 ± 41.8	708 ± 59.7
Clover exudates	66.6 ± 19.9	300 ± 34.5	89.8 ± 15.9	457 ± 65.6	446 ± 172	515 ± 50.5	961 ± 150
Clover cut	35.3 ± 4.48	203 ± 24.2*	67.4 ± 11.9	305 ± 27.0	265 ± 45.6	428 ± 58.8	693 ± 100
AVONA	NS	NS	NS	NS	NS	NS	NS

*Determined by the cutting of the clover shoots after a 100 h labelling period.
NS: main effect or interaction not significant at the P<0.05 level.

Table 5.5. C and N content (mg plant^{-1}) and C:N ratio for plant parts sampled after a 480 h labelling period using the split-root labelling technique to white clover (*Trifolium repens*) and associated ryegrass (*Lolium perenne*). The C and N content for the LC clover roots was not determined. LC= labelling compartment, TC= receiving compartment (mean ± standard error; n=4). One-way ANOVA result comparing the effect of treatments on the resultant plant C and N contents.

N content (mg plant^{-1})				
	CLOVER		RYEGRASS	
	Shoots	Roots TC	Roots TC	Shoots
Control	7.90 ± 1.12	1.89 ± 0.60	1.60 ± 0.64	3.83 ± 0.70
No treatment	6.40 ± 1.68	1.24 ± 0.47	4.16 ± 0.91	5.88 ± 0.86
Clover incorporated	n/a	n/a	3.72 ± 0.35	10.9 ± 0.68
Clover exudates	8.58 ± 1.18	1.56 ± 0.17	3.78 ± 1.39	7.05 ± 0.83
Clover cut	5.84 ± 1.30	1.21 ± 0.14	2.66 ± 0.51	5.24 ± 0.86
ANOVA	NS	NS	NS	NS
C content (mg plant^{-1})				
Control	117 ± 9.47	33.0 ± 9.61	54.7 ± 21.2	119 ± 14.3
No treatment	88.0 ± 16.5	19.9 ± 7.00	118 ± 29.1	176 ± 22.6
Clover incorporated	n/a	n/a	79.4 ± 7.85	183 ± 18.0
Clover exudates	104 ± 12.9	26.0 ± 12.9	104 ± 26.8	206 ± 21.9
Clover cut	76.9 ± 8.80	23.7 ± 2.55	89.1 ± 17.7	170 ± 23.9
ANOVA	NS	NS	NS	NS
C:N ratio				
Control	15.1 ± 1.07	19.0 ± 0.94	33.6 ± 2.62	32.2 ± 2.35
No treatment	15.4 ± 2.56	16.2 ± 1.05	27.0 ± 1.89	30.3 ± 2.33
Clover incorporated	n/a	n/a	21.4 ± 1.25	16.7 ± 0.87
Clover exudates	12.3 ± 0.49	16.5 ± 0.85	31.4 ± 4.52	29.9 ± 2.53
Clover cut	13.5 ± 1.00	19.5 ± 2.62	31.4 ± 2.62	31.2 ± 1.38
ANOVA	NS	NS	NS	P=0.001

NS: main effect or interaction not significant at the P<0.05 level.

For the clover shoots that were incorporated into the soil of the TC with ryegrass, the shoots had a percentage N content of 3.34 ± 0.03% and C content of 39.5 ± 0.09%. There was no difference between the C and N content of the clover shoots grown to be incorporated into the soil and those of the clover plants grown alongside ryegrass in this experiment.

5.4.2. Effect of N pathway on N-transfer from clover-to-ryegrass

Results in this section further address the objective (iii) set out in section 5.2, where the calculations to estimate the N-transfer from clover-to-ryegrass ($Ndft_R$, Table 5.6) agree with the findings in Figure 5.3. Results showed the greatest amount of N-transfer with the clover incorporated treatment which exhibited the highest ^{15}N enrichment in the ryegrass plants. These estimates also show increased N-transfer with increasing ^{15}N enrichment of ryegrass (clover incorporated > no treatment > clover cut > clover exudates). A significant difference was found between $Ndft_R$ with the different treatments ($F_{3,12}$= 16.84, P= 0.000) (yield-dependent equations), where $Ndft_R$ in the clover incorporated treatment was significantly different and higher than the other three treatments, which were not significantly different to each other. Despite this, the values for $Ndft_R$ through yield-dependent equations are much lower than suggested by the high ^{15}N enrichment of ryegrass suggesting significant transfer. This is due to the low amount of clover shoot biomass that was incorporated into the soil (0.7g) along with the fact that this could not be separated from the soil, therefore, transfer to the soil could not be accounted for. Despite Equation 2.26 being chosen as an appropriate calculation in Chapter 4, in this context with a slightly different scenario and parameters of the experiment, yield-dependent calculations (Equation 2.26) may not be a fair representation of the amount of N-transferred to ryegrass. $Ndft_R$ increases for all treatments using yield-independent calculations compared to yield-dependent, apart from the clover exudates treatment where estimates are similar using both calculations. Similarly, the estimate using yield-independent calculations were seen to significantly differ between treatments ($F_{3,12}$= 7.39, P= 0.006). Again, when the clover incorporated treatment is removed from the statistical analysis, a significant difference is found between the other three treatments ($F_{2,9}$= 10.33, P= 0.006), where the clover exudate treatment is significantly lower than the cut clover and no treatment, which were not significantly different to each other.

The amount of N-transferred between plants in this experiment was higher than in previous experiments where the method was developed, for example, application of $CO(^{15}NH_2)_2$ showed

$Ndft_R$ of 3.72% compared to 2.24% in previous experiments (Table 4.8, yield-dependent). However, again no significant difference was found between N-transfer in these two comparable experiments.

Table 5.6. $Ndft_R$ in percentage (%) (proportion of non-legume N derived from the transfer of legume root N) for white clover (*Trifolium repens*) to associated ryegrass (*Lolium perenne*). Yield-dependent estimates were calculated from Equation 2.26, with yield-independent calculations from Equation 2.28, (mean ± standard error; n= 4). One-way ANOVA result comparing the effect of treatments on $Ndft_R$.

	Yield-dependent $Ndft_R$	Yield-independent $Ndft_R$
No treatment	3.72 ± 1.35	8.32 ± 0.71
Clover incorporated	9.34 ± 0.97*	20.5 ± 5.22
Clover exudates	1.15 ± 0.28	1.51 ± 0.34
Clover cut	2.89 ± 0.37	8.85 ± 1.85
AVONA	P=0.000	P=0.006

*Calculated using the fresh mass applied, N content and ^{15}N enrichment of the clover shoot incorporated only. ^{15}N enrichment of the soil was not included in this calculation due to being unable to remove small fragments of shoot.
NS: main effect or interaction not significant at the P<0.05 level.

5.4.3. Effect of treatment on soil amino acids

Results in this section address the objective (iv) set out in section 5.2, where a fairly equal distribution pattern of individual AAs was found for the different treatments, with Asx and Glx generally being present at the highest concentrations (Figure 5.4). However, the clover cut treatment was generally shown to have the lowest concentration of all the AAs, with a significant difference in the total soil hydrolysable AA content being found (Table 5.9, $F_{4,15}$= 3.83, P= 0.026). The clover cut treatment was seen to have a significantly lower total soil hydrolysable AA content than the control and the no treatment, with no significant difference between the other treatments. No significant difference was found between the two-way interaction of treatment and different AAs, however, the concentrations of AAs were seen to significantly different between AAs ($F_{12,246}$= 44.81, P= 0.000) and between treatments ($F_{4,246}$= 16.08, P= 0.000) (Table 4.7). For the interaction of different treatments, the clover cut treatment was seen to be significantly different to all other treatments. However, the other four treatments were not significantly different to each other. A range of significant and non-significant interactions were seen between the concentrations of different AAs, for example, the concentration of Glx was seen to significantly differ from all AAs apart from Asx. While the concentration of Asx was significantly different from Val, Leu, Ser, Pro, Hyp, Phe and Tyr.

Tyr which was present at very low concentrations in the soil was significantly different to all AAs apart from Hyp and Phe (Table 5.8).

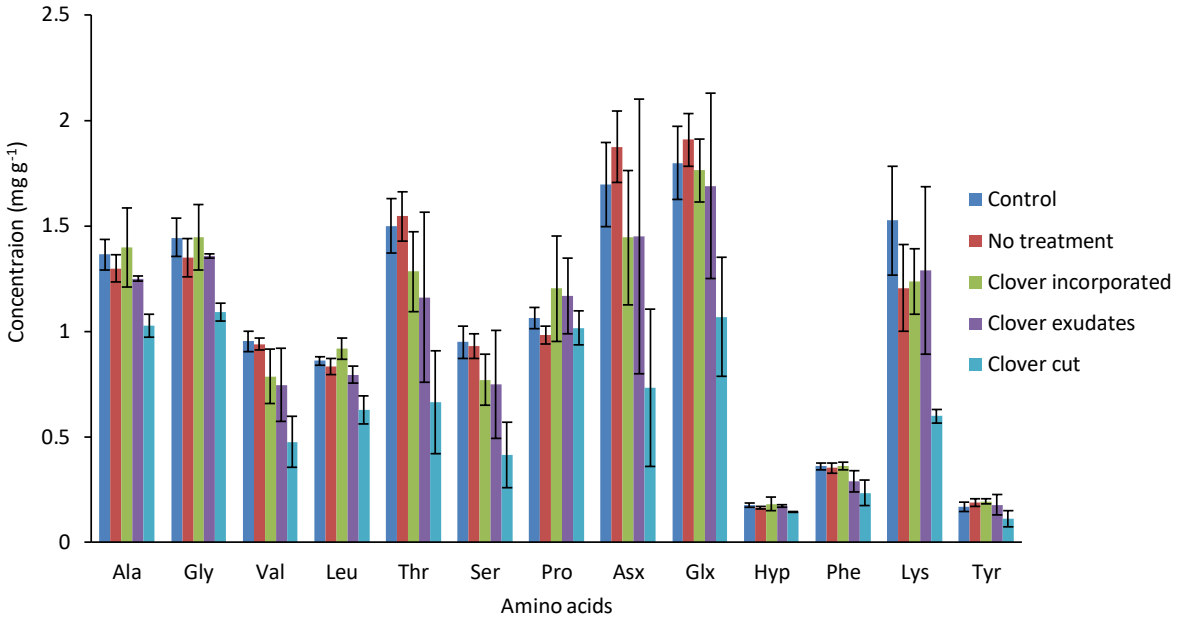


Figure 5.4. Concentration of AAs [mg of AA per gram of sample (mg g^{-1})] in the TC soil after application of ^{15}N-label to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*) using a split-root labelling technique. (mean ± standard error; n=4).

Table 5.7. Statistical results for experiment looking at the effect of different treatments studying exudation and decomposition on the resultant soil AA concentrations.

Statistical test	Interaction	P-value
Two-way ANOVA	AA * Treatment	NS
	AA	P=0.000
	Treatment	P=0.000
Post hoc	Clover cut * All other treatments	P<0.005
	AA concentration interactions	Table 5.8

NS: main effect or interaction not significant at the P<0.05 level.

Table 5.8. Two-way ANOVA post-hoc statistical test for different AAs, showing interactions within a matrix. Statistically significant interactions are denoted by *, whereas the a non-significant interaction at the P<0.05 level is denoted by NS.

	Ala	Gly	Val	Leu	Thr	Ser	Pro	Asx	Glx	Hyp	Phe	Lys	Tyr
Ala		NS	*	*	NS	*	NS	NS	*	*	*	NS	*
Gly	NS		*	*	NS	*	NS	NS	NS	*	*	NS	*
Val	*	*		NS	*	NS	NS	*	*	*	*	*	*
Leu	*	*	NS		*	NS	NS	*	*	*	*	*	*
Thr	NS	NS	*	*		*	NS	NS	*	*	*	NS	*
Ser	*	*	NS	NS	*		NS	*	*	*	*	*	*
Pro	NS	NS	NS	NS	NS	NS		*	*	*	*	NS	*
Asx	NS	NS	*	*	NS	*	*		NS	*	*	NS	*
Glx	*	*	*	*	*	*	*	NS		*	*	*	*
Hyp	*	*	*	*	*	*	*	*	*		NS	*	NS
Phe	*	*	*	*	*	*	*	*	*	NS		*	NS
Lys	NS	NS	*	*	NS	*	NS	NS	*	*	*		*
Tyr	*	*	*	*	*	*	*	*	*	NS	NS	*	

Despite the clover cut treatment having significantly lower soil individual AA concentrations and total soil hydrolysable AA content, no significant difference was found for the soil C and N contents between different treatments (Table 5.9). The percentage N content of the soil was seen to vary between 0.48 to 0.51% and C content varied between 4.46 and 5.00%.

Table 5.9. Mean soil total N (% TN), soil total C (%TC), total soil hydrolysable AA content and total soil hydrolysable content which is N (mg g^{-1}) for the TC soil after application of ^{15}N-label to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*) using a split-root labelling technique. One-way ANOVA result comparing the effect of treatments on %TN, %TC and total hydrolysable AA content in the soil.

	% TN	% TC	Total hydrolysable AA (mg g^{-1})	Total hydrolysable AA N (mg g^{-1})
Control	0.498	4.96	13.86	1.86
No treatment	0.492	4.76	13.68	1.79
Clover incorporated	0.484	4.64	12.99	1.73
Clover exudates	0.495	4.94	10.16	1.36
Clover cut	0.498	4.77	8.21	1.10
AVONA	NS	NS	P=0.026	-

NS: main effect or interaction not significant at the P<0.05 level.

5.4.4. Effect of treatment on incorporation of ^{15}N into soil amino acids

Results in this section address the objectives (v) and (vi) set out in section 5.2, where all treatments applying $CO(^{15}NH_2)_2$ were shown to have elevated AA $\delta^{15}N$ values compared to the control. AA $\delta^{15}N$ values were greatest in the clover incorporated treatment (clover incorporated > no treatment > clover cut > clover exudates > control) (Figure 5.5). For the clover incorporated, clover exudates and clover cut treatments, Hyp was shown to have the greatest ^{15}N enrichment, while for the no treatment Glx was the most ^{15}N enriched AA. For the control, the most ^{15}N enriched AA was Ala.

A significant difference was found between the two-way interaction of treatment and different AAs ($F_{48,259}$= 2.35, P= 0.000) (Table 5.10), showing that when both treatment and different AAs are combined then there is an effect on the AA $\delta^{15}N$ value, but the effect depends on both treatment and the AA. Further analysis of the factors of treatment and AAs showed that a significant difference in the $\delta^{15}N$ values of all individual AAs between treatments existed, where the clover incorporation treatment was significantly different from the rest. Again, if the clover incorporated treatment is removed from the two-way ANOVA analysis, no significant difference was found between the two-way interaction of treatment and different AAs. However, the $\delta^{15}N$ values were seen to be significantly different between AAs ($F_{12,207}$= 2.74, P= 0.002) and between treatments ($F_{3,207}$= 61.69, P= 0.000) (Table 5.10). The $\delta^{15}N$ value of Lys was seen to significantly differ from Leu, Glx, and Hyp, however, all other AAs were not significantly different to each other. For the different treatments, all were seen to significantly differ from each other.

Table 5.10. Statistical results for experiment looking at the effect of different treatments studying exudation and decomposition on the resultant soil AA $\delta^{15}N$ values.

Statistical test	Interaction	P-value
Two-way ANOVA	AA * All Treatments	P=0.000
Excluding clover incorporated treatment in two-way ANOVA		
	AA * Treatments	NS
	AA	P=0.002
	Treatments	P=0.000

NS: main effect or interaction not significant at the P<0.05 level.

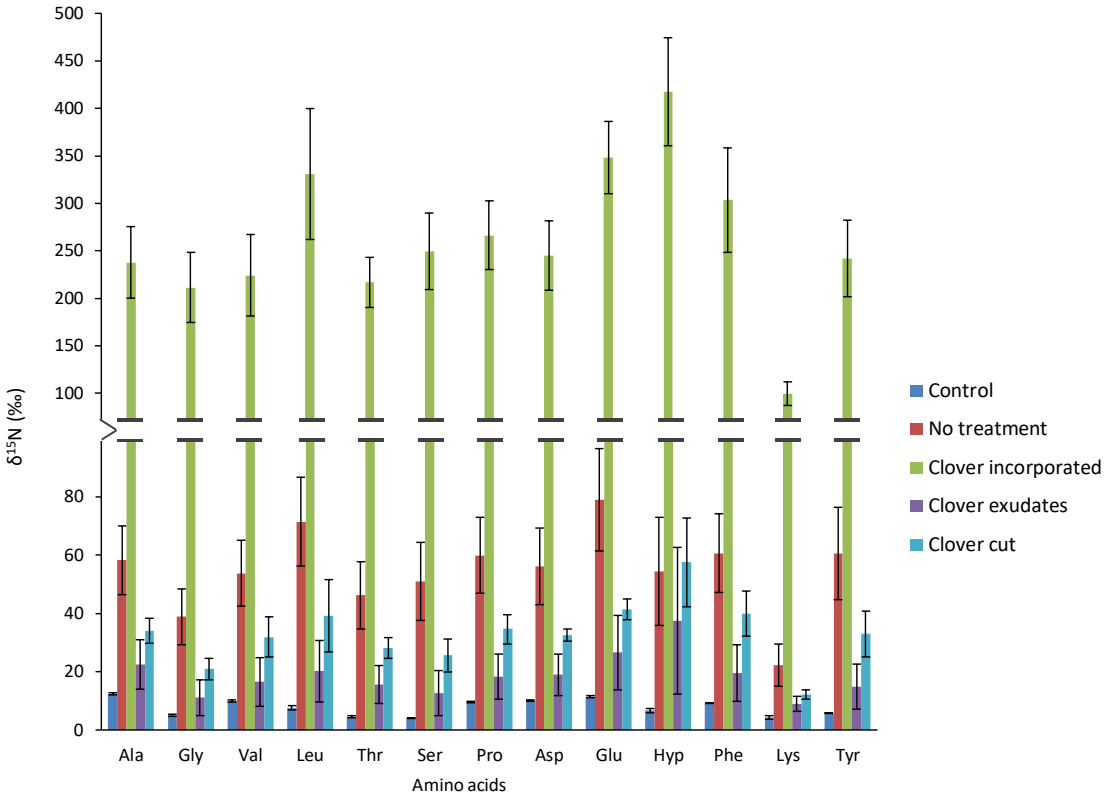


Figure 5.5. $\delta^{15}N$ values of individual hydrolysable soil amino after application of ^{15}N-label to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*) using the split-root labelling technique. (mean ± standard error; n=4).

A different pattern is found with the percentage incorporation of the applied ^{15}N-label into individual AAs, reflecting both the concentration of individual AAs and the $\delta^{15}N$ values. The greatest incorporation was seen into Glx for all treatments, apart from the clover cut treatment (Table 5.11). However, overall there was a very low percentage incorporation of the applied ^{15}N-label into individual AAs, ranging from 0.001 to 0.361%. Despite this, the percentage incorporations were greater than previous experiments, which did not always show incorporation of ^{15}N into all AAs, generally there was at least a 10 times greater incorporation in this experiment, and for Glx this was 100 times in the clover incorporated treatment (Table 4.10 compared to 5.11). Furthermore, by summing the individual AA percentage incorporations, the percentage incorporation into the total hydrolysable AA pool or soil protein pool can be obtained. Unsurprisingly, these values show the greatest incorporation into the soil protein pool for the clover incorporated treatment (clover incorporated > no treatment > clover cut > clover exudates) (Table 5.11). A significant difference was found between the total percentage incorporation into the soil protein pool of different treatments ($F_{3,12}$= 29.21, P=

0.000), where the clover incorporated treatment was significantly higher than the other treatments, which were not significantly different to each other. However, if the clover incorporation treatment was removed from the statistical analysis, no significant difference was found between the treatments.

For the percentage incorporation of the applied ^{15}N-label into the individual AAs, a significant difference was found between the two-way interaction of treatment and different AAs, ($F_{36,207}$= 6.37, P= 0.000), showing that when both treatment and different AAs are combined then there is an effect on the incorporation, but the effect depends on both treatment and the AA. Further analysis of the factors of treatment and AAs showed that a significant difference in the percentage incorporation of all individual AAs existed between treatments, where the clover incorporated treatment was significantly different from the rest. Again, when the clover incorporated treatment was removed from the two-way ANOVA analysis, no significant difference was found between the two-way interaction of treatment and different AAs. However, the percentage incorporations were seen to be significantly different between AAs ($F_{12,155}$= 4.21, P= 0.000) and between treatments ($F_{2,155}$= 40.75, P= 0.000). The clover exudate and clover cut treatments were seen to be significantly different from the no treatment but these treatments were not significantly different from each other. A range of significant and non-significant interactions were seen between the different AAs, for example, Glx was seen to significantly different from Hyp, Phe, Lys and Tyr, however, all other AAs were not significantly different from each other. Similarly, the incorporation into Ala was seen to be different from Hyp, Phe, and Typ. Whereas, the AA Val, Leu, Thr, Ser, Pro, and Asx were not seen to differ from any AA.

Table 5.11. Incorporation of the applied ^{15}N-label into individual AAs (%) for the split-root labelling technique. Maximum values for each treatment are highlighted in bold. Results from statistical tests comparing the % incorporations of different AAs with different treatments are detailed at the bottom of the table.

	No treatment	Clover incorporated	Clover exudates	Clover cut
Alanine	0.061	0.293	0.012	**0.022**
Glycine	0.056	0.334	0.010	0.020
Valine	0.030	0.129	0.005	0.008
Leucine	0.037	0.193	0.007	0.015
Threonine	0.054	0.189	0.010	0.011
Serine	0.038	0.165	0.007	0.008
Proline	0.039	0.218	0.007	0.020
Aspartic acid	0.059	0.236	0.011	0.011
Glutamic acid	**0.081**	**0.361**	**0.017**	0.020
Hydroxyproline	0.005	0.046	0.003	0.005
Phenylalanine	0.010	0.057	0.002	0.004
Lysine	0.029	0.142	0.006	0.006
Tyrosine	0.005	0.023	0.001	0.001
Total incorporation	0.494	2.384	0.098	0.151

One-way ANOVA- Total incorporation	P=0.000
Two-way ANOVA- AA * All Treatments	P=0.000
Two-way ANOVA- AA * Treatments (exc. Clover incorporated)	NS
Two-way ANOVA- AA (exc. Clover incorporated)	P=0.000
Two-way ANOVA- Treatments (exc. Clover incorporated)	P=0.000

NS: main effect or interaction not significant at the P<0.05 level.

5.5. Discussion

5.5.1. Relevance of different N-transfer pathways in the portioning of ^{15}N and N-transfer from clover-to-ryegrass

This chapter is aimed at defining the major processes involved in N-transfer from clover-to-ryegrass as there has been much debate on the contribution of different processes of N-transfer, to which a better understanding is needed to maximise benefits of the association moving towards more sustainable agriculture. This chapter studies a range of different processes by which clover can release N to the soil and where N can be transferred between plants (Table 5.12), as not all processes can be fitted into one single category (Thilakarathna et al., 2016), this made the study more complex. To assist in studying this, the many biotic and abiotic factors which are seen to influence N-transfer were controlled where possible, for example, conducting experiments under greenhouse conditions, using clone plants, and where possible having roots intermingling.

Results from this experiment were promising, especially in terms of land management strategies, showing that maximum N-transfer between plants can be achieved through incorporating clover shoots into the soil. The findings of this experiments are in agreement with previous studies, showing that clover residues can provide an important source of N (Ruz-Jerez et al., 1992; Sparling et al., 1996; Hanyes, 1997; Lupwayi et al., 2006; Dahlin et al., 2011). The different processes which were allowed to co-exist in the clover exudate treatment (Table 5.12) were minimised and the results from this suggest that clover exudates may contribute approximately one-third of all transfer seen, other N-transfer processes must transfer the remainder of N (Figure 5.1). The contribution of each N-transfer pathway is discussed individually.

Table 5.12. Treatments applied in Chapter 5 to study the relative contribution of different pathways in N-transfer from clover-to-ryegrass (*Trifolium repens* and *Lolium perenne*). Each treatment allowed different pathways of N release from clover as well as the subsequent associated exchange mechanisms of N-transfer from clover-to-ryegrass.

Treatment	N release from clover pathway	N exhange processes permitted
No treatment	Death and mineralisation of root and nodules Root exudation	Root-root contact Direct hyphal interaction* Solute diffusion Mass flow
Clover incorporated	Death and mineralisation of shoot material	Solute diffusion Mass flow
Clover exudates	Root exudation	Solute diffusion Mass flow
Clover cut	Accelerated death and mineralisation of root and nodules Root exudation	Root-root contact Direct hyphal interaction* Solute diffusion Mass flow

*Direct hyphal interaction involved the direct connection of plants through common mycorrhizal networks (CMNs), the connection of plant though contact between separate hyphal nets could also occur. It should be noted that all treatments could be subject to mycorrhizal mediated uptake of N, i.e. indirect hyphal interaction.

5.5.2. Effect of incorporating clover on partitioning of ^{15}N and N-transfer from clover-to-ryegrass

The incorporation of clover into the soil facilitated much higher ^{15}N enrichment in the ryegrass and therefore N-transfer than was expected, particularly considering the experimental set-up which minimised the number of N-transfer processes which simultaneously take place within this treatment (Table 5.12). It was demonstrated that an essential part of the N cycle is the release of N locked within cells upon death and decomposition. It is also likely that the conditions for decomposition were favourable in this treatment, as the experimental period was relatively short and the rate of decomposition is fundamental to plant N uptake.

The rate of plant decomposition and mineralisation is affected by many biological, chemical and physical properties which control the rate of plant residue decomposition and therefore mineralisation (Smith et al., 1993; Ambus and Jensen, 1997). The soil environmental conditions for rapid decomposition include: sufficient aeration, adequate soil moisture (~60% of soil pore space filled with water), temperatures between 25 to 35ºC and near-neutral pH (Brady and Weil, 2008). These conditions were almost entirely met through the control greenhouse experiments conducted in this Chapter (Section 2.2.9 and 5.3), which also questions the rate of clover decomposition in a field environment where these factors cannot so easily be controlled.

In terms of using the clover incorporated treatment as a land-use management strategy, there are some factors that need to be considered which influence the physical condition of the residue, which are likely to have contributed to the high amount of N-transfer seen in this treatment. Decomposition rate is influenced through the location of residues, i.e. a faster rate of decomposition may be seen if residues are placed within the soil profile rather than on the surface, due to being in intimate contact with soil organisms and moisture and losing nutrients through leaching less easily (Smith et al., 1993; Brady and Weil, 2008). Therefore, to replicate the high amount of N-transfer seen with this treatment in the field it would be necessary to plough in residues. This is further an important consideration as the incorporation method has been shown to have an effect on N release (Poutala and Hannukkala, 1994; Lupwayi et al., 2006). Furthermore, this would question the effectiveness that clover mulch would have in terms of providing N to an associated crop, although this has been shown to be an effective

method in other studies (Dahlin and Stenberg, 2010a), however, there is a concern over N losses with mulching when leaving residues on the soil surface (Dahlin et al., 2011).

Residue particle size is another important factor which affects decomposition rate, however, there is much conflicting evidence on whether fine or coarse plant material accelerates decomposition. A range of plant materials have been studied, showing that ground plant materials decompose faster and cause greater N immobilization than intact or coarse material, particularly during early decomposition (Sims and Frederick, 1970; Bremer et al., 1991; Li and Mahler, 1995; Angers and Recous, 1997; Ambus and Jensen, 1997), although in the long-term grinding has no effect on N dynamics (Ambus and Jensen, 1997). However, contrasting studies have shown that fine material results in slower decomposition than coarse material (Stickler and Fredrick, 1959; van Schreven, 1964; Jensen, 1994b; Sørensen et al., 1996). In this experiment, clover shoots were finely chopped, mainly to allow incorporation into the small incubation tubes, therefore, further investigation in the field is needed as well as the practical consideration of breaking up clover residues in the field.

Decomposition rate is also determined by the residue quality as a food source for soil organisms (i.e. clover shoots in this experiment), this includes: residue physical condition (Brady and Weil, 2008), and the residues biochemical composition (leaf chemistry), including: C: N ratio, N content, hemicellulose, cellulose, lignins and polyphenol (Chaves et al., 2004, Kriaučiūmienė et al., 2012). Initial N concentrations and availability have been shown to have a fundamental effect on decomposition rates (Melillo et al.,1982). Long-term studies have shown that the net N release from leaf litter is primarily driven by the initial tissue N concentration and mass remaining, irrespective of the climate, soil conditions or organisms (Parton et al., 2007). Furthermore, the C:N ratio has a significant contribution towards litter quality and decomposition rate, where this is effected by the plant residues and the soils themselves. Residue C:N content is important for two main reasons: (i) microbial competition for available soil N is intense when residues with a high C:N ratio are applied and (ii) this determines the rate of decay and N availability to plants (Brady and Weil, 2008). The relationship between decomposition rates and C:N ratios are shown to be negatively correlated (Edmonds, 1980), such as, microbial decomposers can meet their N requirements directly from litter when N concentrations are high (low C:N ratio) (Parton et al., 2007). Whereas, high C:N ratios, can cause a depletion in the soil's supply of soluble N, later resulting in N deficiency in

higher plants (Brady and Weil, 2008), this induces net immobilization where N is converted to microbial biomass or exoenzymes (Parton et al., 2007). In this experiment, clover shoots had an ideal C:N ratio, with an average of 13:1, while the non-legume ryegrass shoots had an average ratio of 28:1 (Table 5.5). Furthermore, it is interesting to see that the incorporation of clover shoots into the soil, and subsequent decomposition, significantly decreased the C:N ratio in the ryegrass shoots (decrease in the roots was also seen but not significantly). The reason for this is unknown, although this is the only treatment in this experiment without some contact/influence of clover roots and potentially clover exudates. However, this result shows that this treatment would also benefit decomposition of ryegrass plants upon death due to the lowering of the C:N ratio and have a wider range of benefits in terms of a land-use management strategy.

Just like the debate over the relative importance of different N-transfer pathways, there is much debate over which factors are most important to the rate of decomposition. The overall controlling factors are: climate, litter quality and the community of decomposer organisms (Coûteaux et al., 1995). On a global scale, Zhang et al. (2008) concluded that litter quality is the most important factor in decomposition, while Aerts (1997) concluded that climate is. However, Aerts (1997) showed that there is a triangular relationship between climate, leaf chemistry and leaf decomposition, in the sense that decomposition is affected by the lignin: N ratio as well as being mediated through an indirect effect of actual evapotranspiration. This means that it is hard to predict if clover incorporation in the field would have such a large role in N-transfer and be able to provide rapid N in the short-term.

In this experiment, the clover shoot material was incorporated into the soil with a sole ryegrass plant (Figure 5.2), therefore, there is potential to further maximise the amount of N-transfer. Studies on decomposition have shown that leaf litter decays more rapidly when placed beneath the plant species from which it originated rather than beneath a different plant species (Gholz et al., 2000), because of specialisation of the soil biota (Ayres et al., 2009). This phenomenon is known as home-field advantage (HFA), although this theory has not been universally supported (Ayres et al., 2009). Studies have found evidence for HFA (Gholz et al., 2000; Ayres et al., 2009; Strickland et al., 2009a, b; Madritch and Lindroth, 2011; Kagata and Ohgushi, 2013), while others have found no evidence for the presence of HFA (Gießelmann et al., 2011; St. John et al., 2011; Keiser et al., 2011; Aponte et al., 2012; Carrillo et al., 2012). Therefore, in the field there could be potential to further maximise decomposition and N-transfer, if clover

and ryegrass were grown in association, which warrants further investigation. Although it is possible that previous land-use of the soils used in this experiment (Section 2.2.1), could have resulted in some "tuning" to clover decomposition.

Results for the clover incorporated treatment also highlighted an additional finding in terms of calculating $Ndft_R$. Results showed that yield-dependent equations may not be suitable when clover and ryegrass are not grown in association with each other, with small but significant amounts of biomass being incorporated into soils. Yield-dependent calculations showed that $Ndft_R$ was approximately three times higher than the no treatment, although the ^{15}N enrichment of the ryegrass was approximately three times higher in the shoots and ten times higher in the roots, suggesting that $Ndft_R$ should be much greater. Further to this, yield-dependent equations $Ndft_R$ for the clover incorporated were calculated using the fresh weight of shoots applied (0.7g), compared to the dry weight used for the remaining treatments. Assuming that a typical water content of a plant is 75% (Brady and Weil, 2008), if this was implied, $Ndft_R$ would equal 3.98%, which would show no significant increase in $Ndft_R$ compared to the no treatment. Therefore, suitable equations need to be chosen on a case to case basis. Although, it should also be noted that when using yield-independent equations, the clover incorporated treatment is shown to only result in double the amount of N-transfer of the no treatment.

5.5.3. Effect of clover exudates on partitioning of ^{15}N and N-transfer from clover-to-ryegrass

As expected low ^{15}N ryegrass enrichment and N-transfer were seen in the clover exudate treatment, which further supported other studies showing that decomposition is more important than exudates in terms of N-transfer (Ta and Faris, 1987; Trannin et al., 2000; Sierra et al., 2007) and that nitrogenous exudation is not a significant N pathway in soil (Hamel et al., 1991a). Results suggest that approximately one third of N-transfer could be as a result of exudate release by clover (Table 5.6). However, this could be an underestimation due to the experimental set-up as the clover exudates treatment in this experiment differed notably from the other treatments due to the need to minimise other plant N-transfer processes taking place. Close root contact has been noted as an important factor influencing N-transfer by exudates due to the fact that there is a higher concentration of N compounds exuded by the root system closer to the roots (Merbach et al., 1999; Thilakarathna et al., 2016) with a positive correlation existing between N-transfer and root contact (Jensen, 1996b). It has also been suggested that

grasses may excrete some substances that stimulate N excretion by legumes, and that this may be another advantage of having grass and legume mixtures (Ta and Faris, 1987). In addition, this treatment removed N-transfer through CMNs. This made it impossible to study the role of exudates with roots in close contact, due to the influence of factors, such as the sloughing-off of root tips and decomposition, therefore, a very similar experimental set-up to that previously used by Paynel et al. (2001a), Paynel and Cliquet. (2003), and Lesuffleur et al. (2007). To ensure that a high concentration of N compounds close to the clover roots was available to ryegrass plants, the sand the clover was rooted in was flushed daily with nutrient solution. Although as seen in Chapter 3, full recovery of AAs is not possible, so it is likely that some ^{15}N compounds would have remained held within the sand.

This experiment cannot show how the role of different N pathways changes over-time, exudates have previously been shown to an important pathway within short-term N-transfer from clover-to-ryegrass and it has been suggested that within the field that exudates have an important role within the first productive years (Burity et al., 1989; Paynel et al., 2001a; Gylfadóttir et al., 2007; Lesuffleur et al., 2013), especially since decomposition of residue takes time. Exudates may have a larger role to play in systems where residues have a high C:N ratio leading to nutrient immobilisation (Jalonen et al., 2009b) as well as the fact that exudates themselves have low C:N ratios, showing that they are a good source of N. Furthermore, root exudation may have a greater role in nutrient limited conditions, due to its direct pathway (Jalonen et al., 2009a). Therefore, it is likely that exudates had a minor role in N-transfer, with the N demand of ryegrass was questioned in Chapter 4 with similar amounts of N being transferred in both directions (clover ↔ ryegrass), showing that N demand was not that great for the ryegrass.

Calculating the amount of N-transferred by exudation is further complicated as the major nitrogenous compound shown to be exuded by most temperate legumes is NH_4^+ (Brophy and Heichel, 1989; Paynel et al. 2001a, 2008; Paynel and Cliquet 2003) which may be retained by negative soil/sand particles thereby decreasing its mobility. Furthermore, NH_4^+ released can be immobilised by microbes, restricting its availability for plant uptake (Jalonen et al., 2009b). Also, clover exudates added into the ryegrass soil would have been diluted by the large soil N pool. Previous studies on exudates have mostly been conducted with sand (Brophy and Heichel, 1989; Paynel et al. 2001a, 2008; Paynel and Cliquet 2003; Lesuffleur et al., 2013). A low uptake by grass of root exudates was also found by Jalonen et al. (2009b), concluding that

microbial immobilisation and soil dilution of exudates were the most likely reasons for this, although all treatments studied in this experiment would have been affected by these conditions. However, increasing the time and distance that N "travels" between plants increases the chance that it is absorbed by microorganisms (Johansen and Jensen, 1996), therefore, this would have been increased in the clover exudate treatment.

5.5.4. Effect of clover cutting on partitioning of ^{15}N and N-transfer from clover-to-ryegrass

The results for the cut clover treatment were not as expected where the clover cut treatment, which aimed at killing the clover plant and accelerating root and nodule decomposition, was seen to result in almost identical N-transfer as the no treatment. Numerous studies have shown the importance of nodule and root decomposition in N-transfer, especially in comparison to other transfer pathways (Ta and Faris, 1987; Trannin et al., 2000; Sierra et al., 2007). The findings were further surprising as a very similar method was used by Johansen and Jensen (1996) whereby pea (*Pisum sativum*) was ^{15}N enriched by a split-root labelling technique with associated barley (*Hordeum vulgare*); 42 days after initial labelling the shoots of the pea were removed, leaving the roots to decompose, final harvesting occurred at 60 days (although only 5 days and 21 days, respectively, in this experiment). Johansen and Jensen's experiments showed no or very small amounts of N-transfer in intact peas, and a 4% increase in N-transfer through shoot removal, however, the increase in N-transfer was only significant when plants were inoculated with mycorrhizal fungi. Similarly, Hamel et al. (1991a), Ikram et al. (1994), Jensen (1996b) and Ayres et al. (2007) found increased N-transfer to the associated plant with death or shoot removal of the legume plant. This finding is typically found since greater amounts of N are lost from dying roots, as well as the fact that shoot removal increased root turnover rate (Ikram et al., 1994).

Experiments in this chapter did not see an increase in N-transfer through cutting clover shoots, and this may be because cutting the clover shoots was not an effective method for killing clover, as within the experimental period the clover shoots had started to re-shoot. Therefore, it may have been more appropriate to cut the clover shoot lower down, removing the link between the labelling and transfer compartments. Repeated defoliation of clover has been shown to increase N-transfer, causing rapid turnover of root and nodule tissue through the death of older plant material as well as greater nodulation of new roots (Butler et al., 1959) also increasing plant

exudation. Defoliated plants often increase C inputs to the soil, leading to soil microbial stimulations which mediate N-mineralisation (Ayres et al., 2007). Belowground decomposition is a slow process compared to the other N pathways such as root exudation and mycorrhizal transfer, with dead tissue needing time to decompose (Ta and Faris, 1987). Other studies reporting positive results of defoliation have had longer experimental periods (Hamel et al., 1991a, Ikram et al., 1994, Johansen and Jensen, 1996, and Trannin et al. 2000). Similarly, N-transfer through decomposition is generally thought to contribute to the later stages of plant growth or in later production years, where increased N-transfer with time and sward age is often found in the field (Burity et al., 1989; Heichel and Henjum, 1990; Høgh-Jensen and Schjoerring, 1997; Jørgensen et al., 1999; Louarn et al., 2015). Often short-term laboratory studies have concluded that direct mechanisms are responsible for N-transfer (Frey and Schüepp, 1992; Ayres et al., 2007). Therefore, time was probably an important factor in the results from this experiment, especially due to the fibrous nature of roots and the higher C:N ratio than that of shoot material, and it is typically found that shoots decompose more rapidly than roots (Sparling et al., 1996; Kriaučiūmienė et al., 2012). Furthermore, often compensatory growth is found in response to defoliation (del-Val and Crawley, 2004; Ayres et al., 2007), short experimental periods were also probably the reason this was not found. However, in terms of land-use management strategies, clover shoot could provide an N source in the short-term and with root decomposition providing N to a subsequent crop later on (Kriaučiūmienė et al., 2012).

Despite some studies showing that leaf removal increases N-transfer, these findings are not always universal. For example, Dahlin and Stenberg (2010a) also found no difference between N-transfer in cut and intact plants [red clover (*Trifolium pratense*) to ryegrass (*Lolium perenne*)], although applying the shoots to the soil surface (mulch) increased transfer. This study showed that a greater proportion of N is transferred when leaf litter can also contribute to N-transfer, this finding further supports the significant role that clover shoot material has in N-transfer.

Predicting belowground transfer is difficult due to the number of different factors which affect it, for example, the amount of N which is transferred between two plants species has been found to depend on the dry matter ratio of the legume and non-legume, C allocation within the non-legume and the root turnover rate (Rasmussen et al., 2007). Therefore, the use of a management strategy which affects the size of the root system and root turnover could also influence N-

transfer, but as the effects are opposing it can be difficult to predict the overall outcome (Dahlin and Stenberg, 2010a). For example, cutting of shoots has been shown to increase senescence and root and nodule turnover (Jarvis and MacDuff, 1989), this can alter the balance of favourable conditions in N-transfer, as it also lowers the root biomass compared to intact plants (Dahlin and Mårtensson, 2008; Dahlin and Stenberg, 2010b).

5.5.5. Effect of the N-transfer pathway on soil amino acids

For most of the different N pathways studied in this experiment, a consistent pattern and distribution of soil AA concentrations were seen. This is also consistent with the AA distribution in previous experiments using $CO(^{15}NH_2)_2$ and the split-root labelling technique. For example, Figures 3.16 and 4.3b, showing high concentrations of Glx, Asx, Ala, Gly, Thr and Lys. Typically, in soils, Asx, Ala, Glx and Gly are present in the highest concentrations (Goh and Edmeades, 1979; Senwo and Tabatabai, 1998; Friedel and Scheller, 2002), therefore, all experiments conducted so far show a slight variation from this with more noticeable concentrations of Thr and Lys. N-transfer pathways were not expected to significantly alter the individual AA concentrations and total hydrolysable AA content, however, the clover cut treatment was found to significantly affect this. The reason for this is mostly likely due to plant stress from removing the clover shoots, which did not completely result in clover death, therefore, it is likely that to support growth, clover had to take up a substantial N supply. This further supports findings from Chapter 4, showing that plants can significantly alter the concentrations of soil AAs under periods of plant stress. Similarly to Chapter 4, the decrease in soil AA concentrations was not accompanied with a decrease in total soil C and N. While no other studies directly linking to this could be found, soil AA concentrations have been shown to significantly decrease over plant growing periods as well as over seasonal changes (Wang et al., 2013; Zhang et al., 2016). Further to this, plant stresses have been shown to affect the influx and efflux of substances in plant roots differently (Macduff and Jackson, 1992).

The ^{15}N enrichments of the soil AAs were as expected from the bulk soil $\delta^{15}N$ values showing the greatest ^{15}N enrichment in the clover incorporated treatment (clover incorporated > no treatment > clover cut > clover exudates > control) (Figure 5.5). Although the percentage incorporation of the applied ^{15}N-label are more useful in providing an indication of the overall fate of the label, taking into account the ^{15}N enrichment and the different AA concentrations. The greatest incorporation was found into Glx, which was also in agreement with previous

findings with the split-root labelling technique and the use of $CO(^{15}NH_2)_2$ (Table 4.10). This finding is unsurprising due to its central role within AA biosynthesis with other AAs being synthesised from it (Section 1.3.2), and the fact that Glx is seen to be exudated in the greatest amount in $CO(^{15}NH_2)_2$ labelled plants (Figure 3.18). Although this experiment cannot tell us which pathway is the dominant process for ^{15}N enriching soil AAs, it is likely to be due to the biochemical cycle of inorganic N and not direct AA exudation. This is because studies on white clover have found larger quantities of NH_4^+ are released than AAs (Paynel et al., 2001a; Paynel and Cliquet, 2003), which can then be assimilated into Glx mainly through the GS-GOGAT pathway, although it can also occur through the GDH pathway (Cabello et al., 2004; Geisseler et al., 2010; Nelson and Cox, 2013).

Results for this experiment showed that the percentage incorporation into Glx was similar to the majority of AAs, apart from that of Hyp, Phe, Lys and Tyr. These AAs were seen to have the lowest ^{15}N incorporations, with Tyr generally having the lowest, this can be related to the further biosynthetic proximity of these AAs from Glx. The biosynthesis of Phe and Tyr (and tryptophan, Trp) involves many steps to produce these aromatic AAs. The first four steps produce shikimate involving the condensation of erythrose 4-phosphate (formed *via* the oxidative pentose phosphate pathway) with phospho*enol*pyruvate (produced from glycolysis) to produce 3-Deoxy-D-arabino-heptulosonice acid 7-phosphate. Following a series of reactions shikimic acid is produced, and then is converted to chorismate in three steps. Chorismic acid is the branchpoint in the pathway and may be converted to Phe or Tyr (or Trp). Similarly, for lysine, there are several steps in its formation in which Asx is firstly synthesised from the transamination of Glx. Asx is condensed with pyruvate to form dihydrodipicolinate (three step process), six further steps including acylation, transamination and decarboxylation results in the formation of Lys (Bowsher et al., 2008; Nelson and Cox, 2013). For Hyp, biosynthesis can only occur from proline (Gerber et al., 1960; Adams and Frank, 1980). Conversions of Hyp have not been found to involve pyridoxal phosphate (Adams and Frank, 1980) which is generally involved in all transamination reactions as well as certain decarboxylation and deamination reactions of AAs (Dolphin et al., 1986). This apparently different pathway results in the lower percentage incorporations. Nevertheless, bacteria have been found to be able to use Hyp as an N source (Adams, 1959; Gryder and Adams, 1969; White et al., 2012; Radkov et al., 2016), although results vary, with some studies finding poor bacteria colony development on Hyp (Halvorson, 1972). Other studies comparing the C substrate utilization of microbial communities on substrates such as glucose, cellulose, lignin, Hyp and gelatine, found Hyp gave

rise to the maximum microbial biomass, community richness and diversity. It was thought these impacts were due to N content, although no other AAs were compared (Schutter and Dick, 2001). Conversely to the low incorporation seen in this experiment, Charteris (2016) found no incorporation of the ^{15}N-label into Hyp, therefore, this was either due to the different bacteria species present in these experiments (which would support findings from other studies which have shown Hyp utilisation) as well as the presence of plants in this experiment, which may have exudated ^{15}N labelled Hyp, which is a major component of plant cell walls (Lamport and Northcote, 1960; Cassab, 1998) as root fragments which may have remained in the soil.

For the other AAs where the percentage incorporations were similar to Glx, the percentage incorporation can mostly be explained by their biosynthetic proximity to Glx, however, this would commonly see the greatest flux into Asx (Figure 5.5a). A larger amount of ^{15}N is seen to be incorporated into Ala than expected (Figure 5.5). However, this could be due to the nature of the soil (i.e. historic background, different microbes), as Charteris (2016) showed that the amount of ^{15}N incorporated into Asx and Ala differed in two soils. Similarly, Charteris showed a higher amount of incorporation into Leu than expected from the known metabolic pathways. In most cases the AA concentrations play a role in vertically ordering the ^{15}N percentage incorporations, where those AAs present in higher concentrations can incorporate more ^{15}N. This is also true for the lower concentrations of Hyp, Phe and Tyr seeing little ^{15}N incorporation.

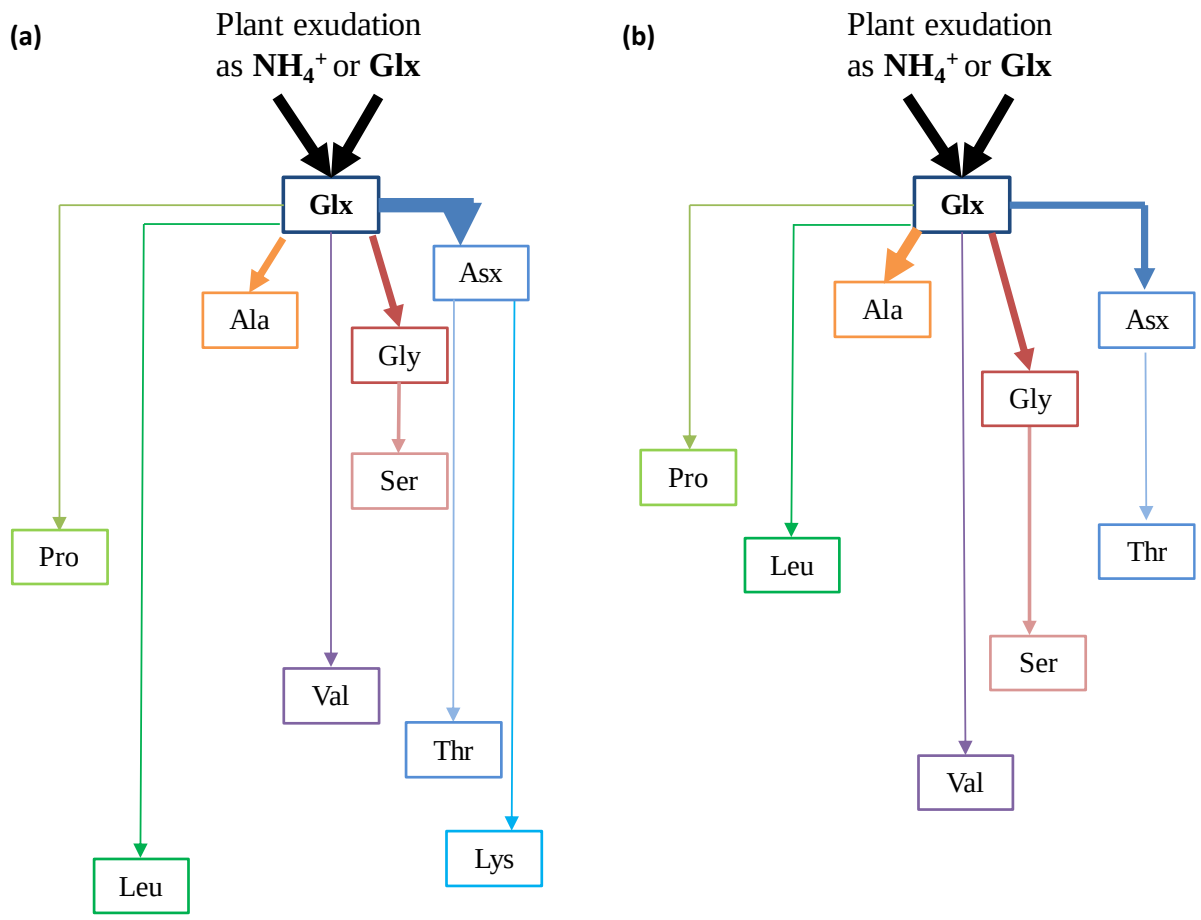


Figure 5.6. (a) Expected biosynthetic pathway of N into the major AAs from their known metabolic pathways (Caspi et al., 2007; Knowles et al., 2010; Nelson and Cox, 2013; Berg et al., 2015; Charteris, 2016). (b) Actual ^{15}N incorporation and routing of N into AAs found to be similar to Glx (average percentage incorporation found in different treatments). Line width represents the total flux of N into each AA and vertical distance from Glx is proportional to the percentage incorporation.

The cut clover treatment was the only treatment not to show preferential routing of ^{15}N into Glx. The percentage incorporation was greatest into Ala, with Gly and Glx having the second greatest percentage incorporation. Although the AA percentage incorporation in the cut clover was not found to significantly differ from the clover exudate treatment, understanding the two-way interactions between the different treatments is complex. Previous studies have shown that defoliation can affect the dynamics of the microbial community structure and nutrient cycling within the rhizosphere, which in turn could alter the incorporation of ^{15}N into AAs. Studies have shown that defoliation can lead to losses in C and N from the plant, increase the soil microbial biomass and bacteria plate counts (Paterson and Sim, 1999; MacDuff and Jackson, 1992; Holland, 1995; Ayres et al., 2007). Interestingly, other studies have reported changes to

the dynamics of the microbial community structure, have reported that not all indicators were affected. For example, defoliation was not seen to change microbial respiration and activity (MacDuff and Jackson, 1992), while Mikola et al. (2001) reported no effect on the microbial biomass, while microbial respiration rate decreased, and Clayton et al. (2005) found that continuous defoliation of both white clover (*Trifolium repens*) and ryegrass (*Lolium perenne*) did not affect the community structure of fungal or bacterial populations. Therefore, it is clear that the effects of plant defoliation on the microbial community are not well understood, although, due to the significant decrease in AA concentration seen in this treatment it is likely to have some effect on the microbial community.

5.5.6. Summary of findings within Chapter 5

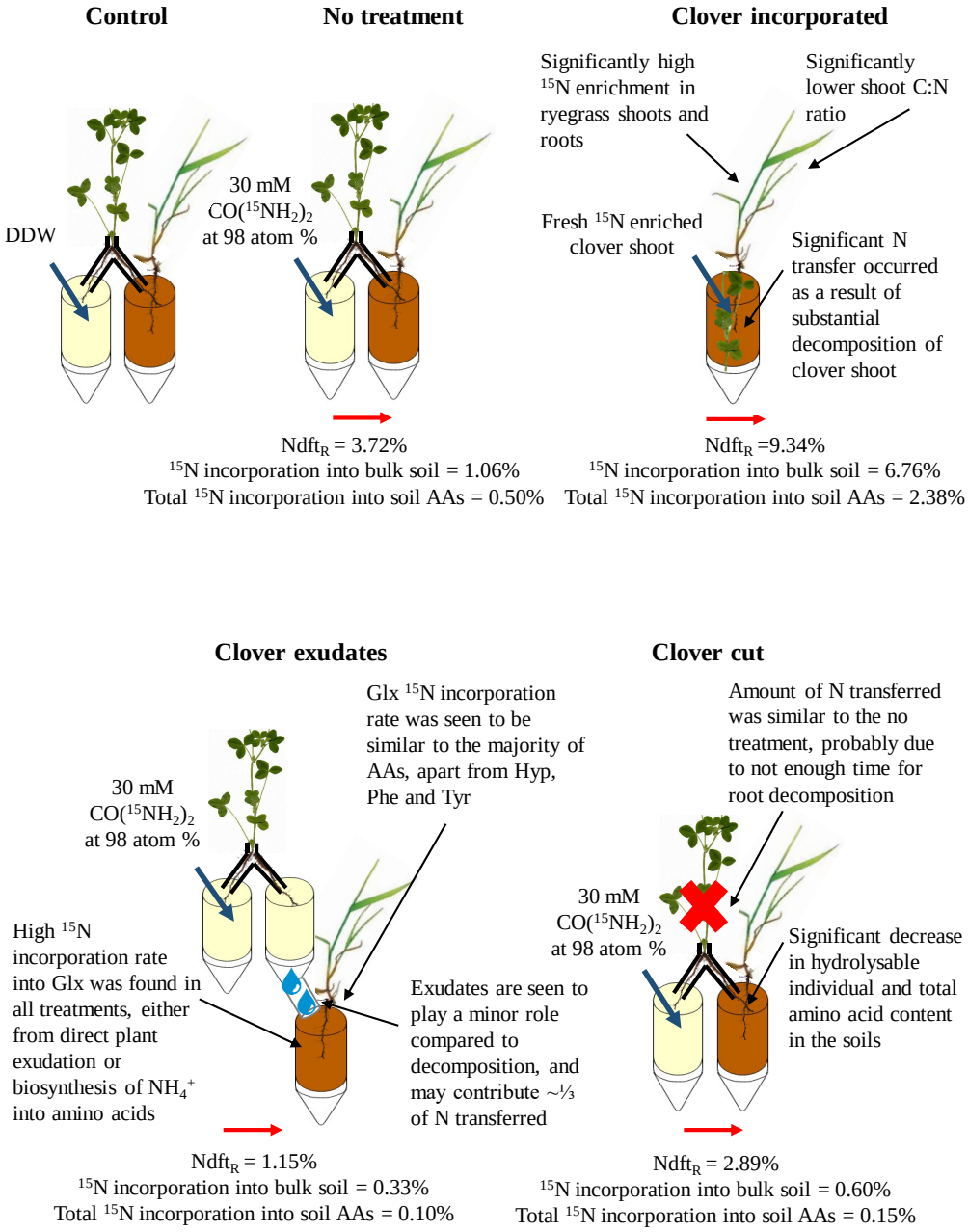


Figure 5.7. Summary figure of experiments conducted within this chapter, which looked at investigating the role of exudation and decomposition in N-transfer from clover-to-ryegrass. Experiments applied a ^{15}N-label to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*) using the split-root labelling technique which was developed through Chapters 3 and 4.

5.6. Conclusion

The methods presented in Chapters 3 and 4 provided a robust technique for investigating different N-transfer pathways from clover-to-ryegrass. This chapter looked at the relative importance of different N-transfer pathways to try and maximise benefits within a field situation to help develop land-use management strategies.

Important specific findings, relating to the objectives set out in section 5.2, include:

(i) Different treatments to the TC were not found to effect the uptake of the ^{15}N-label in clover plants, with no significant difference in $\delta^{15}N$ values of different clover plant parts being found in this chapter compared to Chapter 4 where the method was developed, showing no difference in the uptake of ^{15}N and the repeatability of the method. However, higher $\delta^{15}N$ values of the TC soil, ryegrass roots and shoots were found in comparison to Chapter 4 for all treatments, however, only the $\delta^{15}N$ values for the soil, ryegrass roots and roots in the clover incorporated treatment and no treatment were seen to differ significantly from the control.

(ii) The different N pathways generally did not affect the plant biomass, C and N contents or C:N ratios of plant roots or shoots. Although the clover incorporated treatment was seen to significantly decrease the C:N ratio in the ryegrass shoots, this could have additional benefits to the decomposition rate of ryegrass under different land-use management strategies.

(iii) The different treatments were shown to effect N-transfer (measure as $Ndft_R$), with $Ndft_R$ greatest for the clover incorporated treatment (clover incorporated > no treatment > cut clover > clover exudates) (using yield-dependent equations). A significant difference was found between treatments, where $Ndft_R$ in the clover exudate treatment was significantly lower from the rest. $Ndft_R$ in the treatment only applying $CO(^{15}NH_2)_2$ (no treatment) was similar to the amount of N-transfer previously calculated in Chapter 4. Although findings emphasised the need to choose appropriate expressions to calculate $Ndft_R$ taking into account experimental conditions.

(iv) The clover cut treatment was seen to significantly reduce the concentration of individual AAs and the total soil hydrolysable AA content. This finding shows that plants can significantly alter the soil AA concentrations, and along with findings in Chapter 4, this would suggest that plant stress results in plant uptake of AAs.

(v) The bulk soil and AAs were shown to have the greatest ^{15}N enrichment for the clover incorporated treatment (clover incorporated > no treatment > clover cut > clover

exudates), however, small fragments of the clover shoots incorporated into the soil were not removable so the true enrichment of the soil through mineralisation could not be calculated.

(vi) The percentage incorporation of the applied ^{15}N-label into different AAs was seen to be greatest with the clover incorporated treatments. All treatments generally showed the greatest incoporation into Glx. The percentage incorporation into Glx is seen to differ from Hyp, Phe, Lys and Tyr, however, all other AAs are seen to be similar to each other. This is seen to relate not only to the concentration of these AAs but their metabolic processes, with Glx being central to AA biosynthesis.

The major implications from this chapter were found in addressing objective (vii), finding that decomposition has a greater role in N-transfer between plants than exudation. Results further suggest exudation may contribute to one-third of all N-transferred. In terms of land-use management strategies, in order to achieve maximum clover-to-ryegrass transfer, incorporating clover shoots into the soil would be the most beneficial (i.e. ploughing in clover), especially in the short-term and potentially longer-term benefits could be provided through root decomposition.

Chapter 6

Investigation of the role of soil biota in nitrogen transfer from clover-to-ryegrass

6. Investigation of the role of soil biota in nitrogen transfer from clover-to-ryegrass

6.1. Introduction

Transfer of N from a legume to a non-legume can occur through a number of different pathways, however, there is much conflicting evidence over which mechanisms play a fundamental role in N-transfer, which was examined in Chapter 5. However, the soil environment is dynamic, with many interacting factors as well as having a highly diverse and numerous soil biota community. Not only is this community strongly influenced by plants *via* the 'rhizosphere' effect but it can also shape plant communities and interactions. Soil biota play many important roles within the soil, including in biochemical and nutrient transformations, C sequestration, biological control, bioremediation as well as mediating plant interactions. However, soil biota can be both detrimental and beneficial to the plant (Hunt et al., 1987; Sylvia et al., 2005; Hodge and Fitter, 2013). In terms of improving plant nutrition and performance, nutrient availability in the soil is closely related to microbial activity (Hodge and Fitter, 2013). Soil microbes can form a symbiotic relationship with plants, having a vital role in N_2-fixation as well as nutrient uptake via mycorrhizal fungi (Jacoby et al., 2017), as well as non-symbiotic plant-growth promoting rhizobacteria (PGPR) which are also capable of enhancing nutrient acquisition of N (Richardson et al., 2009).

One method that can be employed to study the many complex biological interactions in soil and to evaluate the role of biota is through firstly reducing the complexity (Mahmood et al., 2014) which is necessary due to the fact that soil organisms are inheritably difficult to study as the majority of microbes are uncultivable (Hodge and Fitter, 2013). To do this, soils are often sterilised in experiments relating to soil biology, there are several methods which can be used in order to achieve sterilisation, such as microwave radiation, gamma irradiation, fumigation (with use of formaldehyde, propylene oxide, chloroform or methyl bromide), or heat treatments (dry or moist) (Alphei and Scheu, 1993; Mahmood et al., 2014). Of these methods autoclaving (moist heat) is found to be one of the most effective methods of eliminating microbes (Razavi darbar and Lakzian, 2007; Mahmood et al., 2014), therefore, it is an important tool for assessing different contributions to N-transfer.

In terms of soil biota native soil, fungi are an important part of any soil due to their capability to fulfil many different roles, such as within ecosystem functioning, vitality and soil biogeochemical cycles. Fungi, such as mycorrhiza, can form a symbiotic association with

plants, colonising the cortical root tissues. Seven basic mycorrhizal types exist with ectomycorrhizae and arbuscular mycorrhizae being the most widespread. The benefits to the plant of mycorrhizal symbioses can be in terms of agronomy (increased yield) or ecologically (improved health or reproductive ability) (Harley and Smith, 1983; Hodge, 2000; Sylvia et al., 2005; van Elsas et al., 2007).

Arbuscular mycorrhizal (AM) fungi occur throughout all plant ecosystems and it is thought that they could infect between 70-90% of plant roots forming mutualistic symbiosis, they show a lack of host specificity varying from herbaceous to woody plants (Bago et al., 2000; Sylvia et al., 2005; Parniske, 2008; Malbreil et al., 2014; Zhang et al., 2017). The characteristic AM structure is the development of a highly branched arbuscule within the cortical cell, which maximises the surface area contact between the plant and the fungus for nutrient exchange (Harley and Smith, 1983; Sylvia et al., 2005; van Elsas et al., 2007). Colonisation by AM have been seen to result in many benefits to plant growth through increased mobilisation, absorption and metabolism of a range of nutrients from the soil, for example, P, N, K, Ca (Marschner and Dell, 1994). Although this benefit is not without cost to the plant, it is estimated that between 10 and 20% of net photosynthates produced by the plant are used by the fungi for the formation, maintenance and function of mycorrhizal structures (Jakobsen and Rosendahl, 1990), with fungi requiring all of its C needs from the host plant (obligate symbionts) (Sylvia et al., 2005). In terms of N, AM have been shown to play an important role, and can uptake and deliver N to plants not only in the inorganic form (NH_4^+ and NO_3^-) but also organic (AAs) (Ames et al., 1983; Bago et al., 1996; Johansen et al., 1996; Cliquet et al., 1997). N can then be assimilated by either the fungi or plant (He et al., 2003). Further to this, for N_2-fixing plants, there is a considerable amount of interaction between rhizobia bacteria and AM fungi with both symbioses acting synergistically. AM benefit rhizobia due to the high P demand of N_2-fixation, legumes typically have coarse roots which are not efficient at extracting P from the soil. However, AM are capable of increasing the availability of P. This leads to enhanced nitrogenase activity, promoting both root and mycorrhizal growth. Furthermore, it has been found that the N and P content of the legume is greater when the combination of inoculates is used (Meyer and Linderman, 1986; Bethlenfalvay, 1992; Sylvia et al., 2005). Furthermore, in terms of N-transfer pathways, AM are thought to mediate N-transfer between legumes and non-legume plants through several means:

(i) Direct transfer of N through interconnecting the roots and serving as a bridge for N-transfer [common mycorrhizal networks (CMNs)]

(ii) Indirect mycorrhizal uptake and translocation, improving the ability of the non-legume plant root system to take up nitrogenous compounds in the soil released by the legume (increased surface area and reduced diffusion length) (Newman, 1988).

It has also been suggested that AM enhance plant exudation or assimilation resulting in more N-transfer (van Kessel et al., 1985; Haystead et al., 1988). Although the involvement of mycorrhizal fungi in N-transfer from legume to non-legume is a controversial topic (Frey and Schüepp, 1992). A number of studies have shown AM to benefit N-transfer between interconnected plants, for example: van Kessel et al. (1985), Haystead et al. (1988), Barea et al. (1989a, b), Bethlenfalvay et al. (1991), Hamel et al. (1991b), Hamel and Smith, (1991), Frey and Schüepp, (1992, 1993), Cheng and Baumgartner, (2004), Xiao et al. (2004), He et al. (2009), Li et al. (2009), Wahbi et al (2016) (as well as references within He et al., 2003). However, this finding is not universal, with other studies concluding that AM are not a pathway for direct N-transfer (Newman and Ritz, 1986; Barea et al., 1989a; McNeill and Wood; 1990; Hamel and Smith, 1991; Hamel et al., 1991b, c; Ikram et al., 1991; Johansen and Jensen, 1996; Hodge and Fitter, 2013; Ren et al., 2017). However, it has been proposed that mycorrhiza provide a framework for the interactions between plant N nutrition which mask the effects seen on N-transfer (Barea et al., 1989a), nevertheless, plants and fungi have been shown to compete for N requirements enhancing their own fitness alongside the plant (Hodge and Fitter, 2010). Further studies on AM have shown that mycorrhizal links increase N-transfer from a dead legume to a non-legume (Hamel et al., 1991a; Jensen, 1996b; Johansen and Jensen, 1996; Muller et al., 2013).

Soil biota comprise a huge range of organisms which could be considered for their role in N-transfer. For example, consideration has been given to typical plant pests, such as weevils which are an important pest in Europe. *Sitona* weevils are commonly found on white clover (*Trifolium repens*), with *Sitona flavescens* being the most common adult weevil (Murray and Clements, 1992). Weevils cause damage to both the shoots and the roots, depending on the exact species and life stage of herbivory species (Murray and Clements, 1992, 1994, 1995, 1998; Murray et al., 1996; Murray et al., 2002). Typically, the larvae feed on and within the root nodules, progressing onto larger roots as they develop, with the adults feeding on the foliage (Bigger, 1930). Due to the fact that the root-feeding larval stage largely remains unseen,

this allows large potential damage to be caused to plants (Murray et al., 2010). Studies have shown that in mixed swards of clover and grass species, damage to the clover root either through mechanical damage or insect herbivory, delivers a positive benefit to the grass species (Hatch and Murray, 1994).

Quite a few studies have been conducted on the effects of weevils on clover, in terms of plant C and N content, N-transfer and uptake to accompanying plants. It has been demonstrated that weevils play an important role within N cycling in grass-clover swards (Murray and Clements, 1992, 1994; Hatch and Murray, 1994; Murray et al., 1995; Murray and Clements, 1998). Hatch and Murray (1994) studied the effect of damaged white clover (*Trifolium repens*) on the N-transfer to intact roots of ryegrass (*Lolium perenne*), finding that significant direct N-transfer to ryegrass only occurred when the clover roots were damaged, resulting in an increase of 37% N content. Similarly, Murray and Clements (1998) also showed a benefit of weevil infested clover plants transferring N to wheat (*Triticum arvense*), concluding that the benefit was most likely to have been seen due to the clover roots becoming impaired in their efficiency to take up N, therefore, reducing competition and making N more available to wheat. However, additional benefits are also gained through the detachment of clover roots by herbivory resulting in its breakdown and N mineralisation at a faster rate than if it was attached (Murray and Clements, 1998), showing longer-term benefits (Cowling, 1982). It has been suggested that the damage to roots and nodules caused by *Sitona* weevils, causes N to 'leak' or be 'flushed' from the legume roots which could provide a pathway for rapid N-transfer between plants (Murray and Hatch, 1994). Furthermore, these findings are not exclusive to weevils, other insect attacks have been shown to increase N-transfer due to slowing plant growth (Giller et al., 1991).

6.2. Objectives

The work presented in this chapter uses the methods developed in Chapter 3 and 4, which looked at methods for introducing a ^{15}N-label into a clover plant and estimating the transfer of N from a legume to the associated non-legume species. This chapter will look at the role different soil biota has in increasing N-transfer between plants, not in terms of their specific role or the processes involved but how transfer can be maximised in an intercropping system in order to help develop land-use management strategies. This is centred around the fact that in natural ecosystems the processes which are involved in conserving productivity and stability

could be combined within agricultural land-use management practices to help develop more sustainable agricultural systems (Wahbi et al., 2016).

This chapter will investigate one of the central hypotheses to this thesis (H2). It is hypothesised that soil biota plays a vital role in mediating the transfer of N originating from clover-to-ryegrass, elimination of soil biota will significantly reduce N-transfer, while enhancement of certain soil biota will enhance N-transfer.

The specific objectives of this work are to:

(i) Compare uptake of ^{15}N in clover and ryegrass using the split-root labelling technique and applying different treatments modifying the soil biology in the transfer compartment.

(ii) Determine whether soil biology modifications in the transfer compartment effects plant growth and plant C and N content in clover or ryegrass.

(iii) Compare N-transfer from clover-to-ryegrass using the split-root labelling technique and applying different treatments modifying the soil biology in the transfer compartment.

(iv) Determine the effect of different treatments to the number of root nodules present on white clover roots.

(v) Monitor the hydrolysable soil AA concentrations for response to the ^{15}N addition to clover plants and soil biology modifications in the transfer compartment.

(vi) Determine and examine the patterns in individual hydrolysable soil AA $\delta^{15}N$ values in response to the addition of $CO(^{15}NH_2)_2$ *via* the split-root labelling technique and soil biology modifications in the transfer compartment.

(vii) Determine the percentage of applied ^{15}N-label incorporated into the total hydrolysable AA pool and whether the different soil biology's effect the total incorporation.

(viii) Comment on the development of new land-use management strategies for the sustainable transfer of N from clover-to-ryegrass.

6.3. Materials and methods

Incubation tubes were set up as described in Section 2.2.10, with one tube of sand acting as the LC and only an additional one tube as the TC. Cuttings of clover and ryegrass plants were taken and allowed to grow in a pot of compost for ten weeks to enable the roots to establish (growing period between April and July 2017, average temperature can be found in Section 2.2.5).

Clover roots were divided between the LC and TC, with the ryegrass being rooted in the TC only. Plants in incubation tubes were moved into the contaminant section of the greenhouse, to reduce the likelihood of whitefly infestations during the running of the experiment (which were a problem in the greenhouse at the time). Plants were left to grow for a further three weeks before the experiment commenced, temperatures for the duration of the experiment can be found in Table 6.1.

The TC in this experiment also acted as the treatment compartment, with five different treatments being studied for their effect on N-transfer. After three weeks of growth within incubation tubes, the substrates were introduced into the LC by injecting (0.25 mL × 4) with DDW for the control ("control") or 30 mM $CO(^{15}NH_2)_2$ at 98 atom % only ("no treatment") or $CO(^{15}NH_2)_2$ to the LC with the addition of treatments applied to the TC of either sterilising the soil ("sterile"), fungi addition ("fungi") or weevil addition ("weevil"), with four repeats per treatment. The sterilised soil was achieved through autoclaving at 123°C for 15 minutes, and clover and ryegrass roots were removed from the incubation tube they had been established in and re-planted in the sterilised soil at the start of the labelling period following thorough root washing with DDW. Fungi [*Rhizophagus irregularis,* formerly named *Glomus intraradices* (Tisserant et al., 2013; Malbreil et al., 2014)] were grown on a vermiculite medium, purchased from Plant Works (Kent), and 1.5g of the growth medium (10% of soil mass) was incorporated on the top of the soil surface of the TC at the start of the labelling period. Additionally, to ensure that the addition of vermiculite didn't influence the growing conditions in any way, the vermiculite was sterilised and similarly added to the other treatments. Weevil eggs (*Sitona* spp.) were acquired as part of another experiment running at North Wyke, Rothamsted Research, in which the eggs were obtained from adult insects caught in the field and held in chambers designed for egg collection (collected May 2017). The collected eggs were kept in the fridge (+4˚C) on filter paper filled petri dishes until required to halt the hatching of eggs (as previously conducted by Murray et al., 1996). Twenty eggs were injected into the soil of the TC at the beginning of the labelling period.

The experiment was halted 480 h after labelling and incubation tubes were sampled. At the end of the experiment plant leaves were cut immediately to halt any further transfer. Subsequently, each incubation tube was deconstructed and plant parts and soils separated. All samples were immediately placed in the freezer, then latterly freeze-dried and then weighed. All plant and soil samples were analysed for bulk $\delta^{13}C$ and $\delta^{15}N$ value determinations (Section 2.3). In

addition, AAs were also extracted from the soils, derivatised and analysed by GC-FID (quantification) and GC-C-IRMS (compound specific $\delta^{15}N$ value determination) (Section 2.4).

Table 6.1. Maximum, minimum and average temperatures (°C) in the containment section of the greenhouse during the experiment.

	Max	Min	Avg
June-17	30.7	15.1	19.1
July-17	29.0	15.5	20.1

6.4. Results

6.4.1. Effect of treatment on partitioning of ^{15}N

Results in this section address the objectives (i) and (ii) set out in section 6.2. The split-root labelling technique was shown to be effective at substantially enriching all plant parts with ^{15}N as well as allowing different treatments to be applied to the soil in the TC (Figure 6.1). The clover shoots and roots in the TC were all noticeably more ^{15}N enriched than the unlabelled control samples. No significant difference was found between the ^{15}N enriched clover plant parts, showing that the different treatments applied did not affect plant uptake of the applied ^{15}N-label in the LC. Furthermore, very similar ^{15}N enrichment in the clover plant parts was found between this study and previous studies where the method was developed (Figure 4.2), with no significant difference between the ^{15}N enrichment of clover plant parts showing the consistency of ^{15}N uptake by clover between studies (Table 6.2).

The TC soil was shown to be ^{15}N enriched compared to the control for all treatments applying ^{15}N, however, no significant difference was found between the $\delta^{15}N$ values of the control and samples where $CO(^{15}NH_2)_2$ was applied in the LC. These results are not too surprising, as the percentage incorporation of the applied ^{15}N-label into the soil was low, between 0.17 and 0.45%, with the lowest percentage incorporation into no treatment soil (Table 6.3). No significant difference was found between the percentage incorporation for different treatments. For an increase in ^{15}N enrichment in the soil to be achieved, ^{15}N transfer must occur from the clover roots. Results showed that the ^{15}N enrichment in the soil represented a low ^{15}N transfer from the clover roots, although the amount was variable between treatments. The greatest amount of transfer from the clover roots to the soil was seen for the fungi treatment (2.15%),

with only 0.33% being seen for the no treatment (Table 6.3). However, no significant difference was found between the different treatments.

However, even though this study found no significant difference in soil $\delta^{15}N$ values when $CO(^{15}NH_2)_2$ was applied or in the $\delta^{15}N$ values of control soils, this study showed the soil to be noticeably more ^{15}N-enriched than in the previous studies (Chapters 3 and 4). For example, a comparable $CO(^{15}NH_2)_2$ treatment having an average of 6.79‰ (Figure 4.2), compared to this study with 12.61‰ (Figure 6.1). Furthermore, there was greater incorporation of the applied ^{15}N-label into bulk soil $\delta^{15}N$ values in this study than in previous comparable studies, for example 0.01% (Table 4.7), compared to 0.17% in this study (Table 6.3).

The results also showed ^{15}N enrichment and therefore N-transfer in the ryegrass roots and shoots, with these samples being more ^{15}N enriched than the control. For the ryegrass roots, the weevil treatment showed the greatest ^{15}N enrichment at 364‰, followed by the sterile > fungi > no treatment, compared to the control at 5‰. However, no significant difference was found between the treatments. For the ryegrass shoots, the greatest ^{15}N enrichment was in the sterile treatment at 111‰, followed by the fungi > weevil > no treatment, compared to the control at 6‰. A significant difference was found between treatments ($F_{4,15}$= 3.190, P= 0.044) (Table 6.2), with the control and the sterile treatment being significantly different from each other, with all other treatments showing no significant difference.

Table 6.2. Statistical results for experiment looking at the split-root labelling technique to white clover (*Trifolium repens*) with different treatments to study the role of soil biota in N-transfer.

ANOVA Interaction	P-value
Clover shoots: All treatments applying ^{15}N	NS
Clover roots TC: All treatments applying ^{15}N	NS
Soil TC: All treatments	NS
Ryegrass roots TC: All treatments	NS
Ryegrass shoots: All treatments	P=0.044

NS: main effect or interaction not significant at the P<0.05 level.

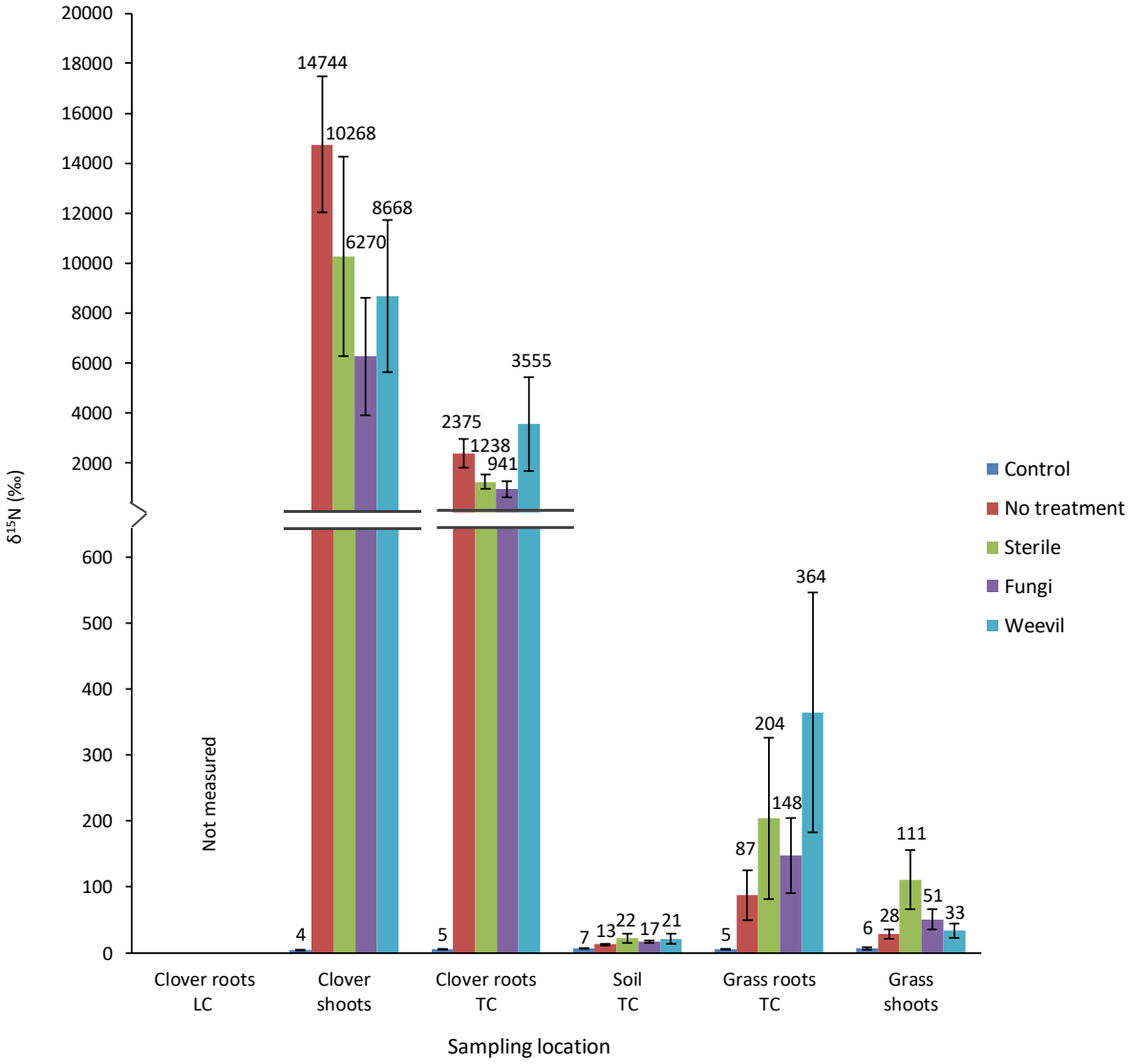


Figure 6.1. $\delta^{15}N$ values of different plant parts after application of the split-root labelling technique to white clover (*Trifolium repens*) and determining uptake in ryegrass (*Lolium perenne*). LC- labelling compartment and TC-transfer compartment (mean ± standard error; n= 4).

Table 6.3. Percentage transfer of the ^{15}N-label from plant derived N to soil [N derived from rhizodeposition (Ndfr)] and percentage incorporation of the applied ^{15}N-label into the bulk soil (%). One-way ANOVA result comparing the effect of different treatments on Ndfr and the incorporation of ^{15}N-label into the bulk soil.

	Ndfr (%)	Incorporation of ^{15}N-label into bulk soil (%)
No treatment	0.33 ± 0.15	0.17 ± 0.04
Sterile	1.30 ± 0.37	0.45 ± 0.19
Fungi	2.15 ± 1.46	0.31 ± 0.07
Weevil	0.56 ± 0.21	0.45 ± 0.23
AVONA	NS	NS

NS: main effect or interaction not significant at the P<0.05 level.

A fairly substantial amount of biomass was produced by clover and ryegrass plants throughout the duration of the experiment (Table 6.4). In comparison to previous experiments, total clover biomass was much greater, for example, the control treatment producing 553 mg, compared to only 166 mg in previous experiments (Table 4.6). For ryegrass, biomass production was not seen to vary so much between experiments, for example, the control treatment producing 436 mg, compared to 408 mg in previous experiments (Table 4.6). The sterile treatment was seen to have the greatest biomass produced by the ryegrass, however, no difference was found between treatments and the biomass produced for individual plant parts. Similarly, the sterile treatment also showed the greatest N content for the ryegrass shoots and roots, and the C content in the shoots. However, for the majority of plant parts no difference was found between the C and N content with different treatments (Table 6.5), apart from the C and N content in the clover shoots. The N content in the clover shoots was found to significantly differ between treatments ($F_{4,15}$= 3.842, P= 0.024), where the control differed from the sterile and no treatment, but all other treatments were not significantly different to each other. Similarly, the C content in the shoots was found to significantly differ between treatments ($F_{4,15}$= 5.000, P= 0.009), where all treatments applying $CO(^{15}NH_2)_2$ were found to differ from the control, but the treatments applying $CO(^{15}NH_2)_2$ were not significantly different to each other. However, no difference between treatments was found for the C:N ratio for any plant part.

Table 6.4. Dry matter (mg plant^{-1}) for plant parts sampled after a 480 h labelling period using the split-root labelling technique to white clover (*Trifolium repens*) and associated ryegrass (*Lolium perenne*). LC= labelling compartment, TC= receiving compartment, (mean ± standard error; n=4). One-way ANOVA result comparing the effect of treatments on the resultant plant dry matter.

	Dry matter (mg plant^{-1})						
	CLOVER				RYEGRASS		
	Roots LC	Shoots	Roots TC	Total	Roots TC	Shoots	Total
Control	120 ± 24.9	370 ± 47.8	82 ± 5.9	553 ± 45.8	99.5 ± 10.6	337 ± 46.0	436 ± 40.3
No treatment	85 ± 16.6	225 ± 25.4	165 ± 86.8	378 ± 26.8	416 ± 115.2	325 ± 64.7	801 ± 88.1
Sterile	87 ± 21.7	220 ± 57.3	56 ± 22.8	349 ± 54.4	346 ± 101.1	470 ± 71.8	816 ± 90.2
Fungi	74 ± 21.4	291 ± 23.1	118 ± 17.6	484 ± 21.2	163 ± 26.7	420 ± 62.2	584 ± 85.7
Weevil	106 ± 46.2	284 ± 49.0	72 ± 16.6	463 ± 71.7	188 ± 63.2	320 ± 37.2	508 ± 79.9
AVONA	NS	NS	NS	NS	NS	NS	NS

NS: main effect or interaction not significant at the P<0.05 level.

Table 6.5. C and N content (mg plant^{-1}) and C:N ratio for plant parts sampled after a 480 h labelling period using the split-root labelling technique to white clover (*Trifolium repens*) and associated ryegrass (*Lolium perenne*). The C and N content for the LC clover roots was not determined. LC= labelling compartment, TC= receiving compartment, (mean ± standard error; n=4). One-way ANOVA result comparing the effect of treatments on the resultant plant C and N contents.

N content (mg plant^{-1})				
	CLOVER		RYEGRASS	
	Shoots	Roots TC	Roots TC	Shoots
Control	11.0 ± 1.08	1.45 ± 0.60	1.23 ± 0.10	6.95 ± 1.36
No treatment	4.23 ± 1.82	2.87 ± 2.07	3.77 ± 1.93	7.13 ± 1.09
Sterile	5.72 ± 1.74	0.83 ± 0.46	5.48 ± 2.73	9.46 ± 0.57
Fungi	8.89 ± 0.66	2.79 ± 0.45	1.58 ± 0.28	7.96 ± 0.96
Weevil	7.52 ± 1.30	1.44 ± 0.32	1.92 ± 0.58	5.53 ± 0.28
AVONA	P=0.024	NS	NS	NS
C content (mg plant^{-1})				
Control	155.9 ± 17.0	21.5 ± 8.4	33.5 ± 4.2	138.6 ± 17.0
No treatment	64.7 ± 26.2	44.6 ± 31.2	103.4 ± 55.3	134.4 ± 30.2
Sterile	83.4 ± 22.2	13.9 ± 7.8	98.7 ± 30.7	190.7 ± 30.1
Fungi	111.4 ± 7.6	40.2 ± 5.7	46.5 ± 7.2	179.1 ± 26.7
Weevil	106.2 ± 19.3	26.8 ± 6.6	48.8 ± 15.3	133.1 ± 14.8
AVONA	P=0.009	NS	NS	NS
C:N ratio				
Control	14.11 ± 0.21	15.18 ± 1.75	27.10 ± 2.44	20.80 ± 1.94
No treatment	15.59 ± 1.00	16.17 ± 0.78	26.84 ± 1.06	20.19 ± 2.55
Sterile	14.92 ± 1.11	16.65 ± 0.36	22.32 ± 3.04	19.89 ± 2.31
Fungi	12.54 ± 0.13	14.54 ± 0.36	29.76 ± 0.90	22.41 ± 1.50
Weevil	14.08 ± 0.31	18.46 ± 1.05	25.25 ± 1.35	24.03 ± 2.32
AVONA	NS	NS	NS	NS

NS: main effect or interaction not significant at the P<0.05 level.

6.4.2. Effect of treatment on N-transfer from clover-to-ryegrass

Results in this section further address the objective (iii) set out in section 6.2, where calculations to estimate the N-transfer from clover-to-ryegrass ($Ndft_R$, Table 6.6) are in agreement with the findings in Figure 6.1. The results show increased N-transfer with applied modifications to the soil biology compared to the no treatment ($CO(^{15}NH_2)_2$ application only). Based on the ^{15}N enrichment of the ryegrass shoot alone (Figure 6.1) results would suggest the greatest N-transfer for the weevil treatment (weevil> sterile > fungi> no treatment). However, calculations taking into account the ^{15}N enrichment in the ryegrass roots and shoots (Table 6.6), show the greatest amount of N-transfer for the fungi treatment (fungi> weevil > sterile> no treatment). Despite this, no significant difference was found between N-transfer of any treatment ($P>0.05$).

The amount of N-transferred between plants in this experiment was lower than previous experiments where the method was developed applying only $CO(^{15}NH_2)_2$ (1.73% compared to 2.24% respectively, Table 4.8). However, again no significant difference was found between N-transfer in these two comparable experiments.

Table 6.6. $Ndft_R$ in percentage (%) (proportion of non-legume N derived from the transfer of legume root N) for white clover (*Trifolium repens*) and associated ryegrass (*Lolium perenne*). Calculated from Equation 2.26. (mean ± standard error; n= 4). One-way ANOVA result comparing the effect of treatments on $Ndft_R$.

	$Ndft_r$
No treatment	1.73 ± 0.52
Sterile	3.10 ± 0.98
Fungi	8.11 ± 4.20
Weevil	4.22 ± 0.81
AVONA	NS

NS: main effect or interaction not significant at the $P<0.05$ level.

6.4.3. Effect of treatment on clover root nodules

Results in this section address the objective (iii) set out in section 6.2, showing that the number of root nodules present on the clover roots in both the sand in the LC and the soil in the TC was variable (Table 6.7). For the LC, a significant difference was found between treatments ($F_{4,15}= 13.974$, $P= 0.000$), with the control having a significantly greater number of nodules than the other treatments, however, all treatments applying $CO(^{15}NH_2)_2$ were not significantly different from each other. For the TC, the weevil treatment had the least number of nodules, however, no difference was found between the treatments.

Furthermore, there was no evidence for the presence of weevils in the weevil treatment, either through recovery or through damage to the nodules. All treatments had both newly formed nodules (which lacked the red leghaemoglobin pigmentation characteristics of active nodules) as well as active nodules, however, this was not quantified between treatments to determine if this was a likely effect of weevil presence.

Table 6.7. Number of root nodules present on clover roots (*Trifolium repens*) in the LC (labelling compartment) and TC (transfer compartment) (count data rounded to the nearest whole number, mean ± standard error; n= 4). One-way ANOVA result comparing the effect of treatments on number of nodules present.

	LC	TC
Control	65 ± 5	38 ± 9
No treatment	25 ± 7	21 ± 6
Sterile	21 ± 7	21 ± 15
Fungi	13 ± 4	35 ± 7
Weevil	21 ± 4	15 ± 2
AVONA	P=0.000	NS

NS: main effect or interaction not significant at the P<0.05 level.

6.4.4. Effect of treatment on soil amino acids

Results in this section address the objective (v) set out in section 6.2, where the control was generally seen to have the greatest concentration of individual AAs, with a reduction seen for treatments applying $CO(^{15}NH_2)_2$. Asx and Glx were present in the greatest concentration in the control and $CO(^{15}NH_2)_2$ treated soils, apart from the fungi treatment, where in comparison these two AAs were depleted, Ala and Gly were present in the greatest concentrations in these soils. Therefore, the results show a slight variation in the distribution pattern of individual AAs, for example, Ala, Gly, Pro and Lys are shown to be at the lowest concentration in the no treatment soil, while Val, Thr, Asx and Glx are at lowest concentration in the fungi treated soil. However, no difference between treatments was found in the total soil hydrolysable AA content (Table 6.9, P>0.05). For the two-way interaction of treatment and different AAs, a significant difference was found ($F_{48,259}$= 1.493, P= 0.031) (Table 6.8), showing that both treatment and different AAs have an effect on the AA concentration, but the effect depends on both treatment and the AA. Further analysis of the factors of treatment and AAs showed that a significant difference in concentration of Val existed with the different treatments, where the control was significantly different from all treatments applying $CO(^{15}NH_2)_2$. Additionally, a significant difference in concentration of Thr resulted from the different treatments, where the control was significantly different from the sterile and fungi treatments.

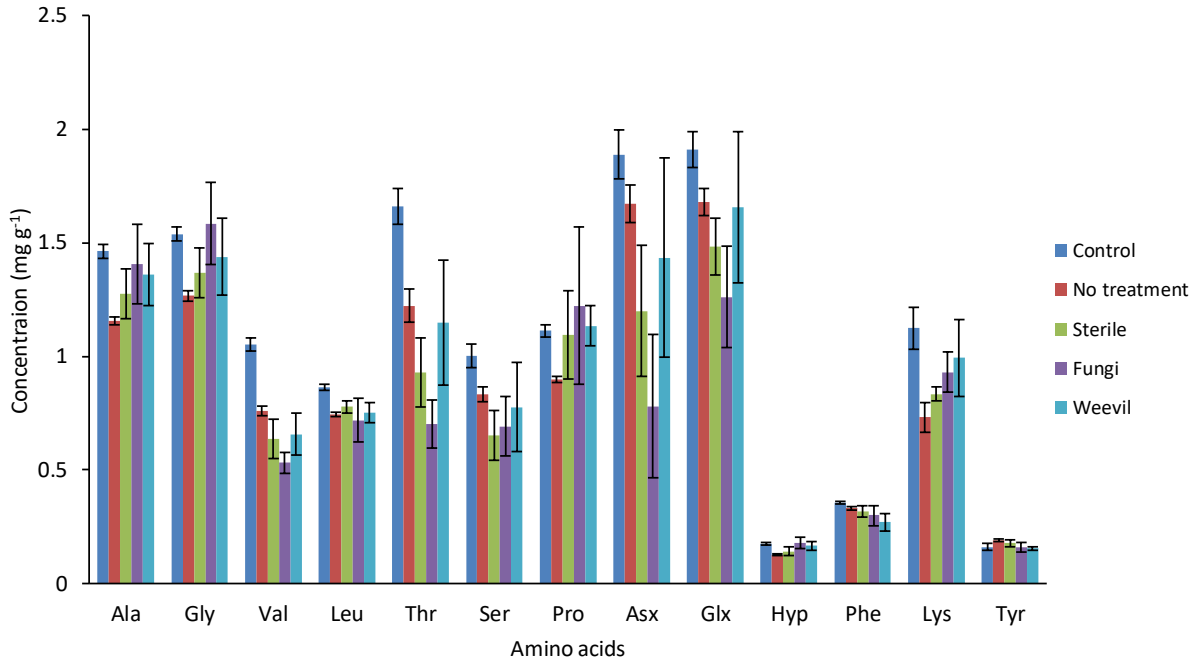


Figure 6.2. Concentration of AAs [mg of AA per gram of sample (mg g^{-1})] in the transfer soil compartment after application of ^{15}N-label to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*) using a split-root labelling technique. (mean ± standard error; n=4)

Table 6.8. Statistical results for experiment looking at the effect of soil biota treatments on the resultant soil AA concentrations.

Statistical test	Interaction	P-value
Two-way ANOVA	AA * Treatment	P=0.031

The percentage N content of the soil was seen to vary between 0.44 to 0.50% (Table 6.9), with a significant difference being found between treatments ($F_{4,15}$= 3.652, P= 0.029), where the control soil was found to differ and have a greater N content than the no treatment soil, however, no difference was found between the other treatments. Overall, in this experiment the N content of the soil was lower than previous experiments, for example, previously the control soil had a N content of 0.51% (Table 4.9), compared to this experiment of 0.48% (Table 6.9). For the C content of the soil results varied between 4.24 and 4.09%, however, no difference was found between treatments. Again, the C content of the soils in this experiment was lower than previous experiments, for example, previously the control soil had a C content of 4.89% (Table 4.9), compared to this experiment of 4.64% (Table 6.9).

Table 6.9. Mean soil total N (% TN), soil total C (%TC), total soil hydrolysable AA content and total soil hydrolysable content which is N (mg g^{-1}) for the transfer compartment (TC) soil after application of ^{15}N-label to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*) using a split-root labelling technique. One-way ANOVA result comparing the effect of treatments on %TN, %TC and total hydrolysable AA content in the soil.

	% TN	% TC	Total hydrolysable AA (mg g^{-1})	Total hydrolysable AA N (mg g^{-1})
Control	0.482	4.64	14.31	1.89
No treatment	0.442	4.34	11.61	1.51
Sterile	0.448	4.49	10.89	1.45
Fungi	0.465	4.52	10.47	1.43
Weevil	0.462	4.53	11.94	1.60
AVONA	P=0.029	NS	NS	-

NS: main effect or interaction not significant at the P<0.05 level.

6.4.5. Effect of treatment on incorporation of ^{15}N into soil amino acids

Results in this section address the objectives (vi) and (vii) set out in section 6.2. All treatments applying $CO(^{15}NH_2)_2$ were shown to have elevated AA $\delta^{15}N$ values compared to the control, generally AA $\delta^{15}N$ values were the greatest with the weevil treatment (weevil > fungi > sterile > no treatment > control) (Figure 6.3). For the weevil, sterile and no treatment, Hyp was shown to have the greatest ^{15}N enrichment, while for the fungi treatment Pro was the most ^{15}N enriched AA. For the control, the most ^{15}N enriched AA was Ala.

No significant difference was found between the two-way interaction of treatment and different AAs, however, the $\delta^{15}N$ values were seen to be significantly different between AAs ($F_{12,259}$= 8.040, P= 0.000) and between treatments ($F_{4,259}$= 17.248, P= 0.000) (Table 6.10). Where the $\delta^{15}N$ values of all AAs were seen to significantly differ from Hyp, however, the remaining AAs were not significantly different to each other. For the different treatments, all were seen to have significantly different $\delta^{15}N$ values than the control, however, all treatments applying $CO(^{15}NH_2)_2$ were not significantly different to each other.

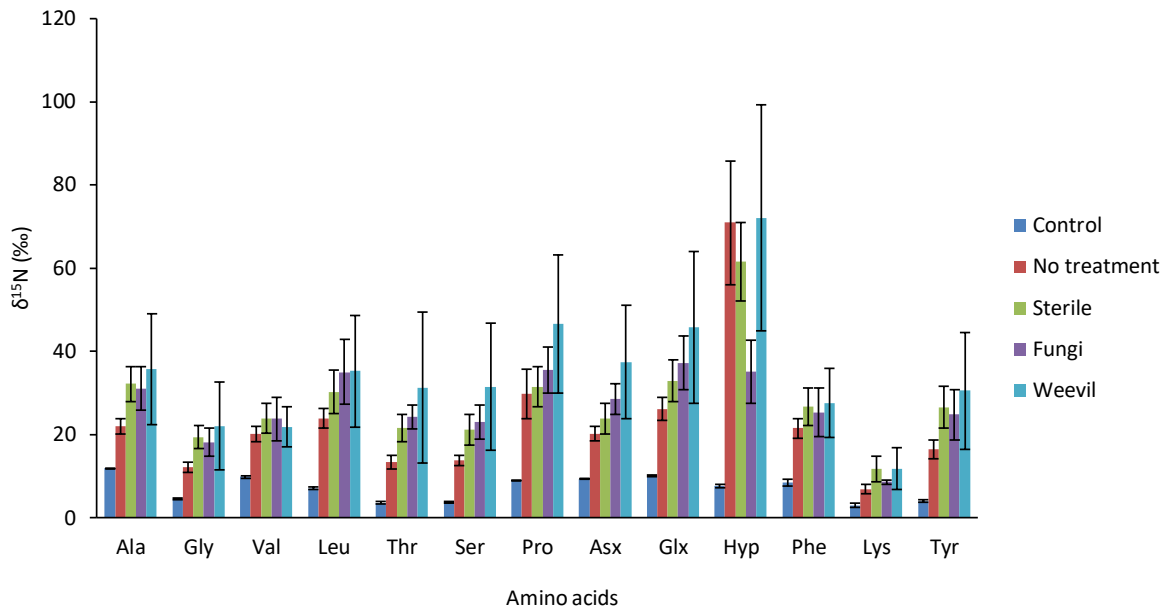


Figure 6.3. $\delta^{15}N$ values of individual hydrolysable soil AAs after application of ^{15}N-label to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*) using the split-root labelling technique. (mean ± standard error; n=4)

Table 6.10. Statistical results for experiment looking at the effect of soil biota treatments on the resultant soil $\delta^{15}N$ values.

Statistical test	Interaction	P-value
Two-way ANOVA	AA * Treatment	NS
	AA	P=0.000
	Treatment	P=0.000

NS: main effect or interaction not significant at the P<0.05 level.

A different pattern is seen with the percentage incorporation of the applied ^{15}N-label into individual AAs, reflecting both the concentration of individual AAs and the $\delta^{15}N$ values. The greatest percentage incorporation is seen into Glx in the no treatment, Ala in the sterile treatment, and Pro in the weevil and fungi treatment (Table 6.12). However, overall a very low percentage incorporation of the applied ^{15}N-label into individual AAs was seen, ranging from 0.0011 to 0.0341%. Despite this, the percentage incorporations were greater than previous experiments, not only showing incorporation into all AAs, but generally seeing over a 10 times greater incorporation (Table 4.10 compared to 6.12). Furthermore, by summing the individual AA percentage incorporations, the percentage incorporation into the total hydrolysable AA or soil protein pool can be obtained. These values showed the greatest incorporation into the soil protein pool for the weevil treatment (weevil > fungi > sterile > no treatment) (Table 6.12),

which is also in agreement with Figure 6.3. However, no significant difference was found between treatments and the percentage incorporation into the soil protein pool.

For the percentage incorporation of the applied ^{15}N-label into the individual AAs, no significant difference was found between the two-way interaction of treatment and different AAs, however, the percentage incorporations were seen to be significantly different between AAs ($F_{12,207}$= 9.272, P= 0.000) and between treatments ($F_{3,207}$= 9.272, P= 0.008). A range of significant and non-significant interactions were seen between the different AAs, for example, the percentage incorporation into Glx was seen to significantly differ from Val, Hyp, Phe, Lys and Tyr, however, all other AAs were not seen to significantly differ from each other. Similarly, the percentage incorporations into Ala and Pro differ from these AAs as well as Thr and Ser (Table 6.11). For the different treatments, the percentage incorporation in the no treatment and weevil treatment were seen to be different from each other, with no significant difference between all other treatments.

Table 6.11. Two-way ANOVA post-hoc statistical test for different AAs, showing interactions within a matrix. Statistically significant interactions are denoted by *, whereas the a non-significant interaction at the P<0.05 level is denoted by NS.

	Ala	Gly	Val	Leu	Thr	Ser	Pro	Asx	Glx	Hyp	Phe	Lys	Tyr
Ala		NS	*	NS	*	*	NS	NS	NS	*	*	*	*
Gly	NS		*	NS	NS	NS	NS	NS	NS	*	*	*	*
Val	*	*		NS	NS	NS	*	NS	*	NS	NS	NS	NS
Leu	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS
Thr	*	NS	NS	NS		NS	*	NS	NS	NS	NS	NS	NS
Ser	*	NS	NS	NS	NS		*	NS	NS	NS	NS	NS	NS
Pro	NS	NS	*	NS	*	*		NS	NS	*	*	*	*
Asx	NS	NS	*	NS	NS	NS	NS		NS	NS	NS	NS	NS
Glx	NS	NS	*	NS	NS	NS	NS	NS		*	*	*	*
Hyp	*	*	NS	NS	NS	NS	*	NS	*		NS	NS	NS
Phe	*	*	NS	NS	NS	NS	*	NS	*	NS		NS	NS
Lys	*	*	NS	NS	NS	NS	*	NS	*	NS	NS		NS
Tyr	*	*	NS	NS	NS	NS	*	NS	*	*	*	*	

Table 6.12. Incorporation into individual AAs (%) of the applied ^{15}N-label for the split-root labelling technique with $CO(^{15}NH_2)_2$ only (no treatment) or $CO(^{15}NH_2)_2$ with sterilised soil, weevil or fungi addition in the transfer compartment soil (TC). Results from statistical tests comparing the % incorporations of different AAs with different treatments are detailed at the bottom of the table.

	No treatment	Sterile	Fungi	Weevil
Alanine	0.0115	**0.0262**	0.0269	0.0295
Glycine	0.0112	0.0243	0.0257	0.0267
Valine	0.0055	0.0066	0.0058	0.0049
Leucine	0.0084	0.0121	0.0144	0.0133
Threonine	0.0080	0.0112	0.0104	0.0116
Serine	0.0069	0.0096	0.0103	0.0112
Proline	0.0144	0.0184	**0.0286**	**0.0341**
Aspartic acid	0.0118	0.0120	0.0108	0.0171
Glutamic acid	**0.0162**	0.0207	0.0233	0.0263
Hydroxyproline	0.0052	0.0048	0.0065	0.0071
Phenylalanine	0.0023	0.0031	0.0031	0.0023
Lysine	0.0035	0.0091	0.0065	0.0099
Tyrosine	0.0011	0.0020	0.0018	0.0019
Total incorporation	0.1059	0.1600	0.1739	0.1960

One-way ANOVA- Total incorporation	NS
Two-way ANOVA- AA * All Treatments	NS
Two-way ANOVA- AA	P=0.000
Two-way ANOVA- Treatments	P=0.008

6.5. Discussion

6.5.1. Modifications to the soil biology effect on partitioning of ^{15}N and N-transfer

Results showed that modifications to the soil biology in terms of sterilising the soil or additions of fungi or weevils, resulted in greater ^{15}N enrichment in the ryegrass plant parts compared to the control or no treatment with $CO(^{15}NH_2)_2$ addition only. As a result, these modifications also resulted in increased N-transfer between clover and ryegrass (Table 6.6), with the fungi treatment resulting in the greatest amount of N-transfer (fungi> weevil > sterile> no treatment). Despite greater amounts of N-transfer being seen, no significant difference was found between treatments using a 95% confidence interval. It is likely that the lack of significant results in this experiment was due to the small number of sample repeats (n=4) and the large amount of variation in the $\delta^{15}N$ values (Figure 6.1) and the percentage $Ndft_R$ (Table 6.6), however, no clear outliers could be identified from the results. Therefore, the apparent differences and the increasing trends in $Ndft_R$ with each treatment are examined in further detail below.

In Chapter 4, the low input of fertiliser to clover plants (30 mM $CO(^{15}NH_2)$) on the dynamics of N_2-fixation and N-transfer was questioned, as it had been shown that N fertiliser application to co-existing clover and ryegrass roots reduces N-transfer from clover-to-ryegrass (Rasmussen et al. 2013). The results in this chapter showed that the application of a ^{15}N-label does have some effect on the dynamics of N_2-fixation, showing a reduction of the number of nodules present on clover roots in the LC in the controls compared with $CO(^{15}NH_2)$ addition. However, the effect on the part of the root system which was studied and used to quantify transfer (TC), did not significantly differ, although a higher number of nodules was present in the control than in the other treatments with $CO(^{15}NH_2)$. Similarly, using a split-root labelling technique, Haystead et al. (1988), found that nodulation was poor in the LC, despite having inoculated the roots, and that nodulation almost exclusively occurred in the compartment which did not receive the labelled N addition.

In these experiments the amount of N-transferred from the legume to the non-legume was quantified in methods developed in Chapters 3 and 4. It has previously been noted that a large proportion of the N-transferred between clover and ryegrass originated from N_2-fixation, and as a result this ^{15}N is unlabelled, complicated the identification of sources and amounts of N-transferred (Murray and Hatch, 1994). Although it is seen that all treatments would be subject

to this problem, and hence would have a minimal effect on identifying the relative contributions to different soil biology modifications on N-transfer.

6.5.1.1. Effect of sterilising the soil

The results for the sterilised soil were not as expected, showing increased N-transfer compared to the no treatment, although not significantly. It was thought that sterilisation would decrease the amount of N-transfer seen between plants due to reducing the belowground processes mediated through root-microbe-soil interactions as well as the fact that autoclaving soils commonly produces inhibitory effects on plants, especially growth (Bowen and Rovira, 1961). However, the slight increase in N-transfer is likely to be due to the reduction in competition between plants and microbes for soil N. This is supported by the fact it has been found that high total microbial C is associated with low ^{15}N-transfer, due to the competitive uptake for nitrogenous compounds (Hamel et al., 1991b). Another mechanism that could have produced the results seen for the sterile soil could arise from the split-root design of the experiment; a developed root system is needed to split the roots between the compartments, microbes therefore would have been present on the root surfaces and re-colonised the soil, this would have introduced a more selected microbe population into the soil, which could have been more beneficial to the clover-to-ryegrass transfer. This can also be seen as an analogue for the home-field advantage hypothesis (HFA).

There has been recent interest in 'home-field effects' and the ability of microbial communities to become 'tuned' to more efficiently processing substrates if they regularly receive them (Austin et al., 2014). This has been studied mostly for plant litter decomposition, whereby, leaf litter decay occurs more rapidly when litter is placed beneath the plant species from which the litter was derived rather than beneath a different plant species (Gholz et al., 2000), as a result of specialisation of the soil biotic community in decomposing litter derived from the plant above it. This phenomenon is known as home-field advantage (HFA), although the support for this theory has not been universal (Ayres et al., 2009). Some studies have found evidence for HFA (Gholz et al., 2000; Ayres et al., 2009; Strickland et al., 2009a, b; Madritch and Lindroth, 2011; Kagata and Ohgushi, 2013), other studies have found no evidence for the presence of HFA (Gießelmann et al., 2011; St. John et al., 2011; Keiser et al., 2011; Aponte et al., 2012; Carrillo et al., 2012). Therefore, it could be viewed that the re-inoculation of the sterilised soil was with a more favourable microbial community that was "tuned" to the N substrates (e.g. NH_4^+ or AAs) released by clover and their transformations. Even if microbes had not been

introduced into the soil from the already established plant roots, bacterial communities are seen to rapidly re-colonise sterilised soils, with the structure of the community developing differing from pre-sterilisation (Marschner and Rumbeger, 2004; Wertz et al., 2007).

However, the relationship between N-transfer, plant growth and re-inoculation is unlikely to be straight forward. Sterilisation is known to alter soil properties [for example: increases and decreases in pH have been reported, cation-exchange capacity (CEC) has decreased, dissolved organic carbon (DOC) and organic N has increased], plant growth and the community structure of newly developed bacterial population have been altered with affects varying greatly with soil type (Bowen and Rovaira, 1961; Salonius et al., 1967; Skipper and Westermann, 1973; Sandler et al., 1988; Alphei and Scheu, 1993; Serrasolsas and Khanna, 1995; Razavi darbar and Lakzian, 2007; Mahmood et al., 2014). These changes are thought to occur due to the release of soluble organic acids from dead microorganisms and humic materials (Razavi darbar and Lakzian, 2007). As a result, soil sterilisation in some studies may also be seen to enhance plant and microbial growth if these conditions are favourable (Skipper and Westermann, 1973) and increased root growth has been found in sterilised soils (Mahmood et al., 2014). However, the effect on plant growth has been seen to vary, with decreases also being reported due to manganese toxicity to plants as a result of microbe elimination which generally transform manganese to higher oxides (Boyd, 1971; Williams-Linera and Ewel, 1984) as well as reduced P availability has also been associated with sterilised soils, due to the elimination of mycorrhizae which increase absorption (Wallace et al., 1973; Sinegani and Jalilvand, 2013).

Mahmood et al. (2014) noted that there have been very few studies which focus on the inherent effects of sterilisation on plant growth and the effect of soil sterilisation on belowground mediated processes through root-microbe-soil interactions, with most studies focusing on effects of microbial inoculations on plant growth. Soil sterilisation has also been found to increase the rhizosheath in wheat seedlings (Mahmood et al., 2014). Rhizosheaths are the layer of sand grains which are tightly bound to the plant root by mucilage, mediated through the interaction between plant root hairs and polysaccharides released by the roots or root-colonising bacteria, these are commonly found in grasses (Bailey and Scholes, 1997; Bergmann et al., 2009). Rhizosheaths have been shown to have several functions, including being associated with N_2-fixation (Bergmann et al., 2009) as well as promoting plant growth in stressful environmental conditions (Amellal et al., 1998; Ashraf et al., 2004). This experiment showed, although not significantly, that ryegrass plants have the highest shoot and total

biomass, N content in the shoots and roots and C content in the shoots, therefore, this could have been due to the promotion of rhizosheaths on ryegrass roots encouraging growth under an induced stressful environment. It is also equally possible that the new microbial population in the sterilised soil could have included bacteria which produced growth promoting substances (Kaci et al., 2005), benefiting ryegrass.

Soil sterilisation has commonly been used to show the value of mycorrhizal fungi within soils, although commonly in these studies sterilised soils are re-inoculated with fungi to study this effect (Thompson, 1990; Van Kessel; 1985; Haystead et al., 1988; Hamel et al., 1991b; Frey and Schüepp, 1992; Wahbi et al., 2016). Sterile soils in this experiment were not purposely inoculated with any organisms, and it can be questioned through which pathways N was transferred. It is not known whether mycorrhizal fungi were able to re-establish themselves in the sterilised soil, providing direct transfer through CMNs, if not this would question the role of mycorrhizal fungi in direct N-transfer. Connectedness of plants *via* mycorrhiza has been shown to increase with time (Merrild et al., 2013). If mycorrhizal fungi had developed, the sterilised soil is likely to have affected their development and abundance. Inoculating sterilised soil with mycorrhizal fungi has been shown to increase root colonisation, however, reducing plant N and P concentrations as well as yield, due to the destruction of native soil microorganisms. Although significant N-transfer *via* mycorrhizal has only been found in non-sterilised soil (Hamel et al., 1991b), therefore, this would question the role of mycorrhizal fungi for N-transfer in the sterilised soil, with mass flow, solute diffusion and root-root contact being other pathways.

6.5.1.2. Effect of fungi addition

The results suggest that AM fungi may improve the efficiency of N-transfer from legume to non-legume, as the addition of fungi was shown to result in the largest amount of N-transferred between plants, however, the increase was not found to significantly differ. Further to this, the fungi addition was seen to increase the ^{15}N enrichment in the receiving ryegrass roots and shoots, compared to the no treatment. This experiment supports evidence from previous studies, showing that AM increase N-transfer between plants and result in increased ^{15}N enrichment in the receiving non-legume plant. Although, this also questions the significance of AM mediated transfer and whether transfer can be large enough to influence plant growth. The method used in this experiment (split-root labelling) has been used successfully in a number of other studies looking at mycorrhizal mediated N-transfer, such as; van Kessel et al. (1985),

Haystead et al. (1988), Eissenstat (1990), Frey and Schüepp, (1993), Johansen and Jensen (1996), showing that this is an effective way to study transfer between plants. This experiment did not look at addressing the likely contribution of different AM pathways, nor to the extent with which the roots were colonized with mycorrhizal or their mycorrhizal status, but whether fungi addition to agricultural soil could enhance plant growth and perturb N-transfer in intercropped systems. Although, it could be seen that AM fungi absorption of N from mineralised roots was a minor role, as also concluded Frey and Schüepp (1992) in short time periods. Furthermore, some studies have shown that inoculation of white clover with AM is necessary for the suitable development of clover, and the natural mycorrhizal potential of some of the soils needs to be reinforced (Barea et al., 1989b). However, generally it is found under a range of plant species that AM hyphal density is greater under intercropping plants compared to singular species (Barea et al., 1989a; Cheng and Baumgartner, 2004; Li et al., 2009; Wahbi et al., 2016).

The evidence in this experiment suggests a small increase in N-transfer between plants with AM addition, however, there are a number of other documented benefits to pasture plants through mycorrhizal infection (Haystead et al., 1998). Despite not being significantly higher than the control, the fungi addition (and weevil addition) were seen to alleviate the decreased N content in the clover shoot, that may have been as a result of $CO(^{15}NH_2)$ addition and changes to the biota community. The fungi addition (although not significant) in this experiment showed that compared to no treatment, fungi addition increased the C and N content in the clover shoots and clover shoot dry matter (resulting in increased total biomass), with these parameters being the highest compared to all treatments with $CO(^{15}NH_2)$ addition. The control was always seen to be greater in these parameters, especially for total clover dry matter, suggesting some effect of $CO(^{15}NH_2)$ addition. However, the addition of fungi was also seen to reduce the C:N ratio of the clover shoots and roots compared to all other treatments (including control), in comparison with the ryegrass where the C:N ratio was increased. Additionally, for the ryegrass, the shoot biomass was increased compared to the no treatment (but not higher than the sterile treatment), with no other plant parameters increased. Typically, AM inoculation has shown significant increases in clover N content and dry matter, which typically suggests that clover is better supplied with N (Haystead et al., 1988) or improvement of P acquisition for N_2-fixation (Meyer and Linderman, 1986; Bethlenfalvay, 1992; Sylvia et al., 2005). Similarly, for non-legume plants increased dry matter production and N matter content has been found (van Kessel et al., 1985). However, similarly to the conflicting evidence

on whether mycorrhizal associations increase N-transfer between plants, a range of different evidence exists in terms of the benefits seen in plants, for example, van Kessel et al. (1985) showed when studying the N-transfer between soybean (*Glycine max*) and maize (*Zea mays*), that maize roots had a significantly lower percentage N in roots which were mycorrhizal infected compared to non-infected, while a similar trend was found for the leaves (however increased dry matter with mycorrhizal fungi resulted in higher N content). Whereas, Frey and Schüepp (1992) studying N-transfer between berseem (*Trifolium alexandrinum*) and maize, found that the dry mass of maize was not affected by mycorrhizal presence, but total N content was higher with mycorrhizal infection. Johansen and Jensen (1996) studying transfer from pea (*Pisum sativum*) to barley (*Hordeum vulgare*) found that the dry weight of pea plant roots and shoots and N content of the shoots was decreased with mycorrhizal infection. Furthermore, Haystead et al. (1988), found that ryegrass grew better with mycorrhizal treatments, although this was not consistent over all experiments conducted. Wahbi et al. (2016) also found a positive effect on the shoot dry weights of the N_2-fixing faba bean (*Vicia faba*) but not in the associated wheat (*Triticum turgidum*). It was also stressed in this study that differing results are found between studies on mycorrhizal transfer, and it is probably closely related to the experimental conditions. It should also be expressed that maybe no significant difference in plant N content and growth was seen in this experiment due to the small compartment size restricting plant growth, and this could hold true for all treatments studied. Furthermore, in studies where plant growth has been shown to be enhanced, it is not known what other nutrients mycorrhizal fungi have helped to increase the availability of, therefore, results generally cannot be attributed to N alone.

The results from this experiment are in agreement with a number of studies which show that AM increase N-transfer between plants, although not significantly (e.g. Hamel and Smith, 1991; Hamel et al., 1991b; Ikram et al., 1991; Johansen and Jensen, 1996), with the percentage of received plant N derived from the transfer (Ndft) being similar to those values reported by Johansen and Jensen (1996) (2-4%). It may be likely, therefore, that the role of AM within soils is to improve the uptake efficiency of nutrients and reduce nutrient losses (Ikram et al., 1994). However, the lack of significant results for the fungi treatment, could be for a number of reasons. Firstly, in this experiment, we did not directly measure the presence and root colonisation by the mycorrhizal fungi, therefore, we cannot say with certain if the roots were colonised. Although this does not affect the outcomes of this experiment other studies which have also evaluated N-transfer pathways have not assessed the mycorrhizal status of plants and

soils (Xiao et al., 2004). It should be noted, however, that N-transfer has been found to be correlated to the amount of soil hyphae, with high N-transfer found with high mycelium density (Hamel et al., 1991b). Secondly, the N demand in ryegrass is an important factor determining the amount of N-transferred, and a concentration gradient is thought to be important for the movement through hyphal links (Francis and Read, 1984; Haystead et al., 1988; Jalonen et al., 2009a). A concentration gradient should exist between legume and non-legumes, especially when the non-legume is dependent on N_2-fixed by the legume. This was questioned in Chapter 4 where equal amounts of N were seen to be transferred in both directions, showing that N demand was not that great for the ryegrass. This observation is further supported by Johnasen and Jensen (1996) who also found that N was transferred in the reverse direction, resulting in an insignificant net transfer of N, with the ability of AM to translocate nutrients in both directions. Further to this, it has been suggested that an N deficient plants and soil may benefit more from mycorrhizal mediated N-transfer (Frey and Schüepp, 1992, 1993; Ren et al., 2017). Interestingly, Bethlenfalvay et al. (1991) also showed that substantial transfer through CMNs only occurred from soybean to maize when soybean was fertilised with mineral N and not when relying on N_2-fixation, further showing the range of complex interactions in N-transfer between plants and questioning if fertilisation has a role in clover-to-ryegrass transfer.

Thirdly, the N source is likely to affect the amount being transported between plants, as it has been found that most mycorrhizal prefer NH_4^+ over NO_3^- (Finlay et al., 1992; Keller, 1996; Putra et al., 1999; Sarjala, 1999), as well as a preference over AA sources (Baar et al., 1997; Sarjala, 1999). Studies have shown that N-transfer is often dependent on the intimacy of roots, (Hamel and Smith, 1992; Xiao et al., 2004), however, this is not likely to have been a contributing factor to the results in this study as roots were in direct contact. Furthermore, Wahbi et al. (2009) noted that it is quite often difficult to compare the differing results and amount of mycorrhizal mediated transfer between studies due to the different plant species used, protocols (split-roots, nylon nets placed in the soil to stop root mixing) and different labelling methods.

Fourthly, mycorrhizal fungi have been shown to effect N_2-fixation, through increases in the nodule number, dry weight, nitrogenase activity in legumes as well as increase overall N_2-fixation (Daft and El-Giahmi, 1974; Smith and Daft, 1977; Kucey and Paul, 1982; Barea and Azcon-Aguilar, 1983; Barea et al., 1989a; Hamel et al., 1991c). Hamel et al. (1991c) found increases in N_2-fixation of up to 55% with mycorrhizal fungi. This could fundamentally affect

results, as an increase in N_2-fixation would result in a dilution of the ^{15}N-label especially in the clover roots, as a consequence the N subject to transfer would have a lower ^{15}N enrichment, reducing the overall ^{15}N enrichment in the ryegrass. The lower ^{15}N enrichment of the clover roots compared to the other treatments with $CO(^{15}NH_2)$ addition suggests that N_2-fixation was increased. Studies have also shown that mycorrhizal fungi effect plants in other ways, with some studies showing that they reduce the ^{15}N loss from legumes, therefore, are able to better conserve the N resource within plant systems (Hamel et al., 1991c). Although, this point can also be disputed as it has been also suggested that the increased N-transfer is found due to AM plants leaking more N (van Kessel et al., 1985).

Furthermore, it is likely that more optimisation of the particular fungi species is needed to find the optimum benefit in terms of increasing N-transfer between plants, with only the addition of one species being considered in this experiment. For example, in a study comparing three *Glomus* species of endomycorrhizal fungi, soybean yield was lowest with plants inoculated with *G.Versiforme*, also having a low tissue P content. It was also concluded that maybe the species of mycorrhizal fungi chosen were less suited to the soybean than the indigenous species present in the soil (Hamel et al., 1991b). Similar conclusions were drawn by Mårtensoon et al. (1998), that N-transfer can be improved in intercropping systems by selecting appropriate plant and mycorrhizal species. *Rhizophagus irregularis* was an appropriate initial fungi species to study, it is one of the most abundant fungi species, and is a useful organism to use within laboratory studies due to the fact it is easy to propagate with different host species and its biology is well-documented (Malbreil et al., 2014) [nb formerly named *Glomus intraradices* (Tisserant et al., 2013; Malbreil et al., 2014)]. It is also noted that mycorrhizae often exhibit little host specificity (He et al., 2003). Other studies have chosen clover (*Trifolium pratense*) as a growth medium for the mycorrhizal inoculum *R.irregularis* (Calvo-Polanco et al., 2014) and it has been used to inoculate both white clover (*Trifolium repens* L.) and ryegrass (*Lolium perenne*) in a number of studies (Ryan et al., 2003; Liu et al., 2011, 2015; Lee et al., 2012; Yao et al., 2014; Malekzadeh et al., 2016; Sut et al., 2016; Xiao et al., 2016). It has also been shown that clover and ryegrass have different preferences for specific AM fungi, questioning the role of CMN's in N-transfer (Zhu et al., 2000). Furthermore, the concentration of *R. irregularis* may need further investigation to improve the outcomes of the study. For example, N-transfer from faba bean (*Vicia faba*) to wheat (*Triticum turgidum*) was studied at three concentrations of *R. irregularis* (0, 1000 or 2000 spores pot^{-1}) with the total amount of N_2-fixed significantly higher at 2000 spores pot^{-1} (due to acquiring P for N_2-fixation) as well as the hyphal density

but transfer of N to the accompanying wheat was greater at 1000 spores pot^{-1}, suggesting that a minimal mycorrhizal network density is needed to optimise transfer. This would also suggest that high hyphal density favours vertical N-transfer (i.e. uptake from the soil) rather than lateral transfer (i.e. from plant to plant through CMNs) (Wahbi et al., 2016). Therefore, showing a trade-off exists between which factors need optimising within mycorrhizal mediated transfer and the desired outcomes required in terms of providing sustainable N through legumes. Although, it should also be noted that N-transfer between plants is not only fungi species specific but also depends on the combination of plants species (Ikram et al., 1994).

Lastly, the difference could be due to the different growth mediums used, representing different nutrient statuses and microbial communities. Frequently studies reporting enhanced N-transfer with AM have been conducted in sterile materials (Van Kessel; 1985; Haystead et al., 1988; Frey and Schüepp, 1992; Wahbi et al., 2016) or with soils with a low N content (Moyer-Henry et al., 2006). Interestingly though, Hamel et al. (1991b) compared sterile and non-sterile soil and found that mycorrhizal enhanced N-transfer between plants was only significant in non-sterile soils. It was noted that using natural soil quite often resulted in ambiguous results due to the range of edaphic and biotic factors. Barea et al. (1989a) only found significant mycorrhizal mediated N-transfer in one out of four P concentrations studied (lowest P addition 125 mg kg^{-1}). Studies conducted in the field have often reported inconclusive results on mycorrhizal mediated N-transfer (Hamel and Smith, 1991, 1992; Hamel et al., 1991c), with Hamel and Smith (1991) noting that their results suggested better P uptake by mycorrhizal plants than interspecific mycorrhizal N-transfer. Hodge (2000) stressed the need to investigate AM under field conditions in order to help fully understand mycorrhizal functioning and the nature of the interactions with other soil biota.

6.5.1.3. Effect of weevil addition

For the weevil treatment no direct or indirect evidence for the presence of weevils could be found. While the number of root nodules on clover roots were lower in the infested plants, this was not significantly different from the other treatments. This finding is surprising as root nodules have been shown to be important in *Sitona* spp. larval development (Goldson et al., 1988; Murray and Clements, 1998; Gerard, 2001; Lohaus and Vidal, 2010). Furthermore, previous studies on the effects of the *Sitona flavescens* weevil on the C and N dynamics of clover have shown that in infested plants there is an 18% reduction in plant dry matter (mostly as result of shoot dry matter reduction), 50% reduction in total plant N, significantly lower C

contents in the shoots, as well as lower C:N ratios (Murray et al., 1994). Similarly, Murray et al. (2002) reported reductions in leaf and root N and biomass in clover. However, no reduction in any of these parameters was seen for clover in this experiment, although, Murray et al. (2002) also showed that loss of root material occurred on a small scale. Although results do vary, with Hatch and Murray (1994) finding that damage to clover roots had no effect on the dry matter yield, similarly, Murray and Clements (1992) found no difference in total root biomass (attached roots plus detached roots caused by weevils) between infected and non-infected plants. It has also been found that legumes may have complementary responses to root damage (Goldson and Jamieson., 1988; Brown and Gange, 1990; Quinn and Hall; 1992, 1996; Murray and Clements, 1992; Gerard et al., 2007) as a result this may be why this experiment did not see any effect on nodule numbers, clover dry matter, C and N contents. It is also likely, that the soil conditions were unfavourable to the weevils resulting in their death after moving out from the nodules, allowing the plant to develop. Further to this, it was also seen that the percentage of plant derived N-transferred to the soil was the lowest for the weevil treatment with the greatest ^{15}N enrichment in the clover roots (although not significantly), this could suggest re-uptake of soil ^{15}N (although damaged roots have a reduced competitive ability for soil N), a reduced amount of N deposition to the soil (although weevil damage causes roots to become 'leaky'), or a reduced N_2-fixing ability (reducing dilution). The last option is likely to be the most plausible, as very similar soil ^{15}N enrichment was seen for all treatments as well as the incorporation of the applied ^{15}N-label into the soil, coupled with the fact that there was a reduced number of nodules, and weevil larvae are known to feed on the nodules making them ineffective.

Evidence of weevil presence couldn't be shown by any indirect effect on the associated ryegrass, in terms of dry matter content and C and N contents. Typically, infestation by weevils will cause leakage of N from clover roots, which in turn will increase N-transfer between plants (Hatch and Murray, 1994; Murray et al., 1996) with no damage being caused to the roots of the non-legume plant (Murray and Hatch, 1994). Although, it has also been concluded in studies that the benefit of the presence of weevils to accompanying plants is due to reduced competition as opposed to the benefit caused by increased N-transfer (Murray and Clements, 1998). Ryegrass with damaged clover roots has shown a significant increase in dry matter content as well as an increase in N content of ryegrass (Hatch and Murray, 1994). Similarly to this study, Murray and Hatch (1994) also found ^{15}N enrichment in ryegrass plants, however, also finding no statistical difference, concluding that this was probably due to the inconsistent nature of the

transfer pathway. Although this study did see a significant increase in the total N content of ryegrass plants with weevil addition, concluding that N-transferred originated from N_2- fixation after labelling the clover plant.

There are a number of possible reasons why the weevil treatment did not significantly affect the amount of N-transfer between plants or cause damage to clover. For example, in the field good clover coverage is needed for the best larval establishment, nodule damage increases with weevil densities, as well as time (field studies have shown reduction in clover yields in the second year of study and not the first) (Gerard et al., 2007). It has also been found that there is no relationship between the clover content and weevil larval populations, or survival rates, although with low clover densities the proportion of infected plants is greater (Murray et al., 2010; McNeill et al., 2016). Furthermore, other than the close relationship between root herbivores and plant host, there are additional factors determining their presence and success. Soil moisture has been found to be the most important factor effecting root herbivores and this is closely linked with soil temperature. Other factors which influence root herbivores include: oxygen and CO_2 composition, soil pH, soil texture and structure, predators presence (nematodes, carnivorous insects) and pathogens (Barnett and Johnson, 2013). Further to this, the presence of mycorrhizal fungi has been shown to help protect the plant from root herbivores (Currie et al., 2011; Johnson and Rasmann, 2015). Therefore, this could have alleviated the negative effects of the *Sitona* weevils and as discussed previously the presence of mycorrhizal fungi was not confirmed (Section 6.5.1.2).

Weevil addition resulted in $Ndft_R$ of 4.22%, and this was not found to differ from the non-infected treatment (1.73%). As discussed, numerous studies have described negative effects of *Sitona* weevils on clover plants as well as positive effects of increased N-transfer between plants. Although it should be noted that the majority of these experiments were either conducted hydroponically in a soil-free growth medium (Hatch and Murray, 1994; Murray and Hatch; 1994) or on a low N status soil (Murray and Clements, 1998). When soil is completely eliminated N-transfer quantities are artificially enhanced, as it eliminates processes that usually compete for available N. Furthermore, Murray and Clements (1998) concluded that direct N-transfer may not occur with greater N availability. Similarly, sterilised soils were often used in studies looking at mycorrhizal mediated N-transfer (Section 6.5.1.2). The experiments in this chapter used an agricultural soil, where the use of legumes to reduce the dependence on N fertilisers is being investigated (Section 2.2). Despite conducting greenhouse experiments, the

amount of N-transfer is more likely to closely represent the actual amounts of N being transferred in the field compared to using soil-free growth mediums. Therefore, this could be the major reason behind the differences between this experiment and previously reported findings. Further to this the agricultural soil being studied is not of low N status, showing the importance of developing land-use management strategies on similar substrates to those in the field.

6.5.2. Effect of soil biology on soil AAs

The distribution of soil AAs varied between the treatments, with all treatments having a lower total hydrolysable AA content compared to the control (although not significantly). Previous experiments in Chapters 3 and 4 (Figure 3.16, 4.3 and 4.6), have also shown slight variations between the concentrations of soil AAs with different treatments. Although no significant difference in the AA concentrations between different treatments was seen, it is likely that the modifications to the soil biota did influence the AAs released by the plant, uptake, the structure of the soil microbial community and the utilisation of AAs. A number of studies have demonstrated a range of these effects, but not specifically altering the distribution of soil AAs. For example, AM have been shown to increase plant uptake of certain AAs, especially those which are relatively rare in protein, neutral or positive-charged AAs (Whiteside et al., 2012). While weevil infested plants have been shown to alter the composition of exudates, particularly showing that in exudates there are greater amounts of Ser and Asp, as well as overall greater amounts of amino N being exudated by infested plants (Murray et al., 1995).

For the $\delta^{15}N$ values of the individual AAs and percentage incorporation of the applied ^{15}N-label, values were shown to increase from the control > no treatment>sterile> fungi> weevil. It is interesting to see that the $\delta^{15}N$ values of the individual AAs do not seem to follow the same pattern of ^{15}N enrichment as the bulk soil (which would generally be expected) (control > no treatment> fungi > weevil>sterile). The ^{15}N enrichment and lower percentage incorporation into the AAs seen for the sterile treatment compared to the bulk soil is however not unsurprising. This is probably due to the reduction in the microbial community leading to a decreased microbial assimilation rate and competition for resources. Although, with reduced microbial assimilation, it is surprising that the percentage incorporation is greater than the no treatment, although again this is most likely due to the specialisation of the microbial community (Section 6.5.1.1).

For the fungi addition, the results for the ^{15}N enrichment in the soil as well as the high level of ^{15}N enrichment seen in the individual soil AAs would suggest that direct transfer from the clover to the ryegrass plants did not occur (i.e. interconnected roots, CMNs) as this would assume that N being transferred does not enter the soil. Mycorrhizal mediated transfer may still be taking place but indirectly involving the release of N through the soil, such as; hyphal to soil, soil to hyphal. This result would also suggest low N demand by the ryegrass, resulting in N not being taken-up from the soil, as well as the role of concentration gradients in mycorrhizal transfer which were discussed in Section 6.5.1.2.

For the weevil addition, the bulk $\delta^{15}N$ values, AA $\delta^{15}N$ values and percentage incorporation into AAs were greater than non-infected plants, this finding is in agreement with previous studies, which have shown greater amounts of amino N to be exudated by infested plants (Murray et al., 1995). The greater amount of amino N exudation is thought to be related to the vascular damage and general disruption to the transport system caused by weevils (Powell and Campbell 1983). Direct AA exudation is the mostly likely reason for finding of higher AA $\delta^{15}N$ values and ^{15}N incorporation into AAs than in non-infected plants, as non-infected plants have been found to exude more NH_4^+ than infected plants (Murray et al., 1995), which would then be available for microbial assimilation into AAs. Therefore, it could be that in this study NH_4^+ exudation from non-infected plants was not so high or that microbial assimilation of inorganic N into AAs was low, and that the findings are as a result of direct plant AA exudation.

It is also interesting to see that the percentage incorporation of the applied ^{15}N-label into different AAs varied with different treatments. A significant difference was found between the incorporation into individual AAs in the no treatment and weevil addition, but not into the total hydrolysable soil AA/soil protein pool. The results for the no treatment are in agreement with previous findings with the split-root labelling technique with the use of $CO(^{15}NH_2)_2$, showing the highest incorporation into Glx (Table 6.12 compared to Table 4.10) as well as in Chapter 5, due to its central role within AA biosynthesis with other AAs being synthesised from it (Section 1.3.1). However, other treatments varied, with the sterile treatment showing the highest incorporation into Ala, and Pro for the fungi and weevil treatment. Interestingly, the percentage incorporations into Ala, Gly, Leu, Pro, Asx, and Glx were not found to significantly differ from each other. For the majority of these AAs, this can be explained from their biochemical pathways, previous studies have shown that ^{15}N incorporation into Asx is related to its close biosynthetic proximity to Glx, with commonly the greatest flux of ^{15}N seen into

Asx from Glx (Knowles et al., 2010). Although this finding was not apparent in this experiment with the incorporation into Asx being either the third to fifth highest depending on the treatment (see discussion in Section 5.5.5 and Figure 5.6). Following Asx, the total flux of N and the biochemical proximity to Glx is seen to be into Ala and then Gly (with Ser being synthesised from transfer on N from Gly), this is then followed by Pro. The high incorporation into these AAs, related to their biosynthesis through fewer intermediates, compared to the other AAs. These results also show the decoupling of C and N cycles, where the transfer of N between AAs is seen to be more important in the incorporation of ^{15}N than the origin of carbon skeletons which form the main precursors for the synthesis of AAs, as shown in Section 1.3.1 and the expected routing in Figure 5.6 (Knowles et al., 2010). From this, it would be expected that Val would have a high percentage incorporation and not be similar to Leu as found in this experiment. Additionally, it should also be noted that generally the AAs present in the highest concentrations in soils, typically resulted in the highest ^{15}N incorporation, furthermore, the concentration is seen to vertically order the AAs by the percentage incorporated. This finding was commonly found by Charteris (2016) when looking at applying a ^{15}N-label directly into soils. For soils, typically, Asx, Ala, Glx and Gly are present in the highest concentrations (Goh and Edmeades, 1979; Senwo and Tabatabai, 1998; Friedel and Scheller, 2002), which is generally in alignment with the findings in this experiment with some higher quantities of Thr (especially in control where ^{15}N incorporation cannot be determined).

For the percentage ^{15}N incorporations, although the incorporation into the "central" AAs was not seen to vary, slightly different patterns in incorporation into other AAs were seen (Table 6.12. Although small this is not too surprising, as the applied treatments are found to induce different plant exudation patterns (as discussed below) or change the microbial assimilation rate and microbial community (especially in the case of the sterile treatment). For the sterile treatment, autoclaving is known to alter soil properties, in respect of N, it increases the availability, exchangeability and extractable amounts, including significant increases in NH_4^+, NO_3^- and total extractable N (Eno and Popenoe, 1964; Salonius et al., 1967; Skipper and Westermann, 1973; Lopez and Wollum, 1976; Alphei and Scheu, 1993; Serrasolsas and Khanna, 1995; Razavi darbar and Lakzian, 2007). Therefore, it would be expected that this would influence the assimilation pathway into the AAs. Similarly, for the fungi treatment, within the soil mycorrhizal roots and soil microorganisms compete for nitrogenous compounds (Cliquet et al., 1997), therefore this would influence the ^{15}N incorporation. Although, similarly biochemical pathways exist, for example, AM plants metabolism of NH_4^+ has been found to

be by the GS-GOGAT pathway, assimilating it into Glu and Gln before into other AAs (Cliquet and Stewart, 1993; Johansen et al., 1996). Although, within the AM, Arg has been found to be the major form of N synthesised and stored in the extraradical mycelium of AM as well as transported to the intraradical mycelium, however, transfer to the host plant cells has been shown to be mainly as NH_4^+ from Arg breakdown (Govindarajulu et al., 2005; Jin et al., 2005). The effect of this cannot be directly seen in this experiment, as Arg was not identified and quantified using the protocol in this experiment (Section 2.4), despite the fact that the AM fungi is likely to have been present in the soil analysed. Further to this, AM have been shown to influence transcription pathways related to plant N uptake and metabolisms, therefore influencing plant metabolic processes (Govindarajulu et al., 2005; Jin et al., 2005; Guether et al., 2009; Saia et al., 2015). Additionally, AM fungi have been shown to decrease root concentrations of most compounds involved in all metabolic pathways, especially AAs. For example, Saia et al. (2005) showed that within durum wheat roots (*Triticum durum* Desf.) AM fungi down-regulated key pathways relating to primary metabolism, such as AA biosynthesis, (especially involving Ala, Gln, Asn and Phe), showing a shift from biosynthesis of common AAs to GABA. Therefore, if AM could induce an effect on the infected roots, it is thus likely that AM could affect AA biosynthesis in soils, whether directly or through the induced changes to the root which would also influence exudation of ^{15}N compounds.

It is also likely that there was some change in the ^{15}N incorporation with fungi addition as it has been noted that AM can modify root exudation either through the fungi acting as a C sink for the photoassimilate and/or through hyphal exudation, leading to changes in both the quality and quantity of exudates (Hodge, 2000). The reported effect on root exudates varies. It has been suggested that AM enhance plant exudation (van Kessel et al., 1985; Haystead et al., 1988; Qiao et al., 2015; Wahbi et al., 2016). Although, decreased root exudation has also been reported due to reduced root membrane permeability and the ability of fungi to reduce loss of N resources from the plants (Graham et al., 1981; Dixon et al., 1989; Hamel et al., 1991a; Marschner et al., 1997). Similarly, others have reported no change to exudation and no direct alteration of the equilibrium of AAs in the root zone (Azaizeh et al., 1995; Cliquet et al., 1997; Marschner et al., 1997). Marschner et al. (1997) reported that the effect on exudation depends on the particular species present. Nevertheless, despite the conflicting evidence on the effect of AM on exudation, mycorrhizal roots and soil microorganisms compete for nitrogenous compounds released by the roots, therefore they could play a substantial role in the utilization

of previously released AAs (Cliquet et al., 1997), therefore, influencing the incorporation of ^{15}N into the AAs.

The results suggest no significant change in the biosynthesis of new AAs and the total soil protein pool, understanding the full extent of the applied treatment on the soil biota and functioning of the soil is complex, especially when looking at the interaction between fungi and soil microbes. This is because the clover responses to AM addition is unlikely to be due to AM alone, but involves other microbes and interactions amongst soil microflora (Hamel et al., 1991b). In terms of creating and developing sustainable land use management strategies, it is important to study a larger range of indicators to determine the functioning of soil processes. For example, it is known that mycorrhizae and microorganisms interact on many different levels, therefore, it is crucial to acknowledge the effects of these interactions on other beneficial soil microbes (Linderman, 1998). Considering the fact that in the field AM ecology is likely to differ, for example, in an agricultural setting where crops are removed the fungus is continuously having to re-establish itself, as well as the impact of fertilisers and pesticides on AM formation (Hodge, 2000). It is known that there are opposing factors amongst mycorrhizal fungi and other soil microflora, it has been suggested that these are important factors to consider when looking at the factors affecting N-transfer between mycorrhizal plants. This is further emphasised by the fact that, high mycelium density in the soil is related to low soil microbial C, and that it has been found that regulation of the soil microbial population plays a greater role in facilitation of N-transfer than mycorrhizal fungi (Hamel et al., 1991b).

It is important to consider the implications of applying fungi inoculations in the field to increase N-transfer using a range of biological indicators before making modifications to the soil biota community. This is because there are contrasting interactions between AM and soil microorganisms. It has been found that root mycorrhizal colonisation results in the development of a unique rhizosphere microbial community, termed the mycorrhizosphere, due to the energy-rich carbon flow from the plant to the fungi (Harley and Smith, 1983; Sun et al., 1999; Hodge, 2000Johansson et al., 2004; Sylvia et al., 2005; Artursson et al., 2006; van Elsas et al., 2007). However, evidence for the impact of mycorrhizosphere communities is inconsistent, with increases, decreases and no change being found in soil after AM colonisation (Ames et al., 1984; Meyer and Linderman, 1986a, b; Klyuchnikov and Kozhevin, 1990; Olsson et al., 1996; Andrade et al., 1997; Marschner et al., 1997; Edwards et al., 1998; Green et al., 1999; Burke et al., 2002). Where AM did not affect the total number of microorganisms

present, they did affect the specific groups of microorganisms present, finding more facultative anaerobic bacteria in the rhizosphere of AM colonised clover (*Trifolium Subterranean* L.), showing that the microbial equilibrium had been altered (Meyer and Linderman, 1986a). However, the effect seen may be due to which fungus has colonised the roots, for example; Schreiner et al. (1997) studied soybean (*Glycine max* L.) with three different AM fungi and observed differences in the bacteria groups (Gram-negative or Gram-positive). *Glomus mosseae* had the lowest Gram-negative bacterial populations as well as the greatest amount of external hyphae, *G. rosea* the lowest Gram-positive bacteria and *G. etunicatum* having the highest Gram-negative, Gram-positive bacteria and total bacteria. Similar results were also reported by Andrade et al. (1997) but not by Olsson et al. (1996) for *Glomus* species. Therefore, the varying results reported on the influence of AM on soil microorganisms are likely to be due to a combination of factors, such as nutrient availability and type of AM fungus. Furthermore, not only do AM fungi effect the soil microbial community, the soil microorganisms influence the AM fungal development and symbiosis establishment (Hodge, 2000) and equally this interaction, can be positive (e.g. Azcon-Aguilar and Barea, 1985; Azcon-Aguilar et al., 1986; Meyer and Linderman, 1986b; Bagyaraji and Menge, 1978; Gryndler et al., 1996; Aponte et al., 2017), neutral (Edwards et al., 1998) or negative (e.g. Ross, 1980; Tylka et al., 1991; Wyss et al., 1992; McAllister et al., 1994, 1995).

6.5.3. Summary of findings with Chapter 6

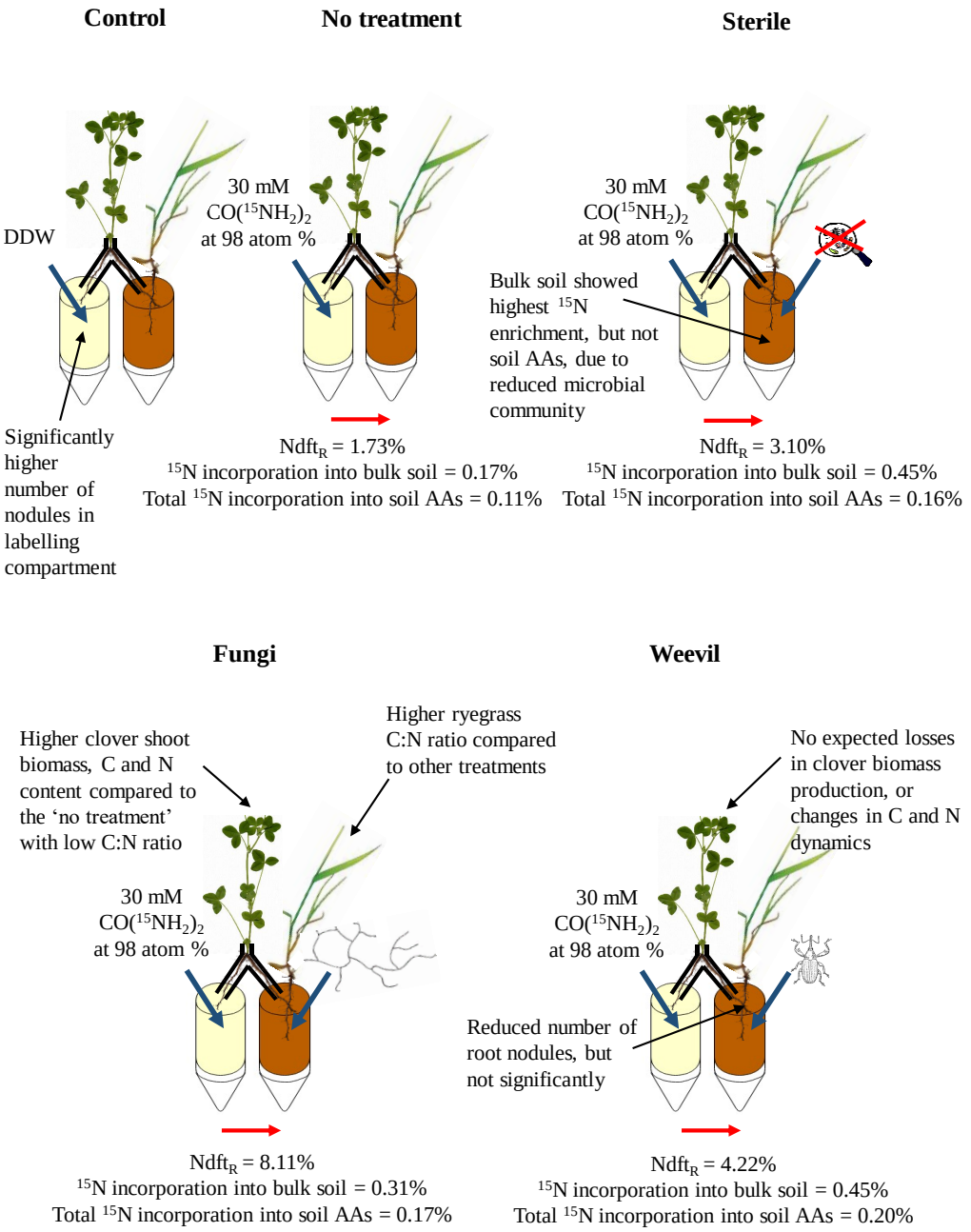


Figure 6.4. Summary figure of experiments conducted within this Chapter, which looked at investigating the role of soil biota in N-transfer from clover-to-ryegrass. Experiments applied a ^{15}N-label to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*) using the split-root labelling technique which was developed through Chapters 3 and 4. Clover plants were either labelled with DDW for the control $CO(^{15}NH_2)_2$ only ('no treatment'), or $CO(^{15}NH_2)_2$ with sterilised soil, weevil or fungi addition.

6.6. Conclusion

The methods presented in Chapters 3 and 4 provided a robust technique for investigating the role of soil biota in N-transfer from clover-to-ryegrass. This chapter looked at ways in which the N-transfer from clover-to-ryegrass could be increased within a field situation in order to help develop land-use management strategies.

Important specific findings, include:

(i) Different treatments to the TC were not found to effect the uptake of the ^{15}N-label in clover plants, with no significant difference in $\delta^{15}N$ values of different clover plant parts being found in this chapter compared to Chapter 4 where the method was developed, showing no difference in the uptake of ^{15}N and the repeatability of the method. Further to this, higher $\delta^{15}N$ values of the TC soil, ryegrass roots and shoots were found in comparison to experiments conducted in Chapter 4, however, for the soil and ryegrass roots no significant difference was seen compared to the control. For the ryegrass shoots, only the $\delta^{15}N$ values for the sterile treatment were seen to significantly differ from the control.

(ii) Different treatments modifying the soil biota were not found to affect the plant biomass, C and N contents or C:N ratios of plant roots or shoots.

(iii) The different treatments were shown to effect N-transfer (measure as $Ndft_R$), with $Ndft_R$ greatest for the fungi treatment (fungi > weevil > sterile > no treatment). However, no significant difference was found. A similar amount of N was transferred in these experiments as calculated previously in Chapter 4.

(iv) Application of $CO(^{15}NH_2)_2$ was seen to reduce the number of clover root nodules compared to the control in the LC, although no effect was seen in the TC soil, which is the part of the rooting system under study. This result shows that treatment did not effect the number of nodules.

(v) Different treatments modifying the soil biota community were not found to effect the total or individual hydrolysable soil AA concentrations.

(vi) For the individual AA $\delta^{15}N$ values Hyp was the most ^{15}N enriched AA. For the AA $\delta^{15}N$ values the most important finding was the different enrichment patterns between bulk and individual AA $\delta^{15}N$ values for the different treatments. The bulk soil was shown to have the greatest ^{15}N enrichment for the sterile treatment (sterile > weevil > fungi > no treatment), however, similar percentage incorporations of the ^{15}N-label into the bulk soil were found for the sterile and weevil treatments. When the ^{15}N enrichments

of individual soil AAs were studied the greatest enrichment was seen for the weevil treatment (weevil > fungi > sterile > no treatment), showing that the soil sterilisation reduced microbial assimilation into AAs, as well as supporting previous studies showing greater amino N exudation from weevil infested plants.

(vii) The percentage incorporation of the applied ^{15}N-label into different AAs is seen to vary, with the incorporation into Glx being similar to Ala, Gly, Leu, Ser, Pro and Asx but significantly differing from the other AAs. This is seen to relate not only to the concentration of these AAs but their metabolic processes.

The major implications from this chapter were found in addressing objective (viii), in terms of developing new land-use management strategies. This chapter altered the soil biota community with the aim of perturbing N-transfer, the results in this chapter stress the importance of studying soil biota in agricultural soils as well as using a large range of biological indicators in developing land-use management strategies due to the wider implications that alternating the soil biota may have on other processes especially seeing as many conflicting studies exist. Aside from this another major implication from this chapter was questioning the role of direct transfer through mycorrhizal fungi (CMNs) due to the ^{15}N enrichment of soil as well as questioning the N demand of ryegrass.

Chapter 7

Overview and recommendations for future work

7. Overview and recommendations for future work

7.1. Overview

The overall aim of this thesis was to improve understanding of the pathways of N-transfer from clover, transportation through soils and the uptake by ryegrass in order to contribute to developing land-use management strategies thereby decreasing the reliance on synthetic fertilisers. Furthermore, due to the lack of knowledge regarding the cycling of organic N in soils, because of its complexity and heterogeneity, much of organic N remains unidentified apart from proteins consisting of AAs which present 20-50% of total soil N (Schulten and Schnitzer, 1998; Friedel and Scheller, 2002; Roberts and Jones, 2008) as well as amino N being a major component of legume exudates. Therefore, a compound-specific isotope approach was used alongside bulk ^{15}N isotopes to expand current knowledge of N-cycling through the soil AA pool.

The initial chapters of this thesis provided insights into the most appropriate method to use to study N-transfer between clover and ryegrass within laboratory studies, showing that even though methods to introduce a ^{15}N-label to plants have already been established, they are not necessarily appropriate for the plant species being studied or the aims of the study. This demonstrates the importance of preliminary investigation and range-finding experiments. For example, plant uptake of N compounds varies, resulting in greater ^{15}N enrichment of clover with $CO(^{15}NH_2)_2$ than $^{15}NH_4{}^{15}NO_3$. This finding has wider implications since NH_4NO_3 is currently the most commonly used fertiliser in the UK (Defra, 2017). However, the plant exudation pattern of clover was seen to change with the application of $CO(^{15}NH_2)_2$ compared to the control and $^{15}NH_4{}^{15}NO_3$, resulting in greater Glx exudation compared to Pro, although the importance of this in a wider agricultural context is not known. Further to this, the difficultly of achieving elevated ^{15}N enrichment above background values when studying N-transfer between two plant systems was demonstrated, showing that it was necessary to use a concentration of 30 mM at 98 atom %, despite the concern of ^{15}N discrimination and isotopic fractionation that may occur at high ^{15}N enrichment levels, as well as potential effects of the down-regulation of N_2-fixation and number of nodules on clover. However, it was found that when using a split-root labelling technique the effect on root nodules was only significant in the labelling compartment, therefore, it was assumed that the effects would have been less important for the part of the root system that was under study, as well as being necessary to study N-transfer between plants.

Unexpectedly, the initial investigation questioned the most commonly used leaf-labelling technique, which was seen to result in lower plant ^{15}N enrichment than that of the root-labelling technique meaning N-transfer could not be detected. In addition the concentration of soil AAs was significantly affected. For these reasons, the leaf-labelling technique was not seen as appropriate to address the aims of this study, and a split-root labelling technique which follows the natural pathway of assimilation was used.

Surprisingly, quantification of N-transfer from clover-to-ryegrass ($Ndft_r$) (without any additionally manipulations to the system) resulted in lower values than expected. $Ndft_r$ over all experiments averaged 2.67%, this is most likely due to the relatively short-term study, soil N status and ryegrass demand. This is reinforced by the fact that N-transfer in the reverse direction was seen to be similar. Ryegrass in this experiment did not show any signs of N deficiency (yellow leaves, stunted growth), therefore, it is likely that greater N-transfer would be seen in N limited systems and over-time as the soil N pool becomes depleted.

The developed method was then adapted in later chapters to examine the different pathways which are known to contribute to N-transfer between legumes and non-legumes. As a starting point, the role of exudation and decomposition in N-transfer were examined. The difficulty of eliminating other N-transfer processes was demonstrated, but overall results proved that decomposition has a greater importance in N-transfer than exudation, which is in agreement with current views on N-transfer pathways (Section 5.1), however, no estimates of the relative size of these different N pathways exist. Results (Section 5.4.3) suggest that exudation contributes approximately one-third of all N-transferred, it can be assumed that in below-ground transfer pathways without any other interaction (i.e. mycorrhizal hyphae) decomposition is responsible for the remainder of N-transferred. It was also shown that N-transfer may be enhanced by the incorporation of clover residues, accelerating the death and decomposition process. Although, it is important to achieve synchrony, as increased N inputs increases N cycling and potential N losses (Ledgard, 2001), therefore, this should be applied in periods of maximum demand. A further advantage of incorporating clover residues was the lowering of the C:N in ryegrass shoots, which could be beneficial when pastures are ploughed before replanting allowing rapid decomposition and N release. The importance of above-ground processes in N-transfer (which are commonly neglected) was emphasised, especially as the greatest amount of N-transfer was seen where clover shoot material was incorporated,

showing that this more easily decomposed material can provide considerable amounts of N in the short-term.

The role of soil biota in mediating N-transfer as well as the possibility of enhancing N-transfer between plants was examined in Chapter 6, although no significant differences were found, results did offer insight into developing land-use management strategies (Figure 7.1). Results from the AM fungi addition treatment were the most promising, showing the greatest increase in N-transfer compared to the treatment receiving no modifications. However, the mechanisms for this enhanced N-transfer can be questioned for this treatment, due to the levels of ^{15}N enrichment seen in the soil, as well as the higher level of rhizodeposition (Ndfr) seen compared to other treatments (Table 6.3), therefore, suggesting direct N-transfer not to be a major pathway. The lack of adverse effects on plant growth C and N content were further surprising with the addition of *Sitona* weevils, demonstrating the ability of plants to compensate for below-ground herbivore attack. This could also be of benefit in an agricultural setting allowing clover to re-establish itself after herbivore attack. Furthermore, care in interpreting studies carried out in sterile or hydroponic mediums and the agronomic significance of these results was expressed in Chapter 6, showing the relevance of conducting experiments in agricultural soils and producing meaningful results to develop land-use management strategies.

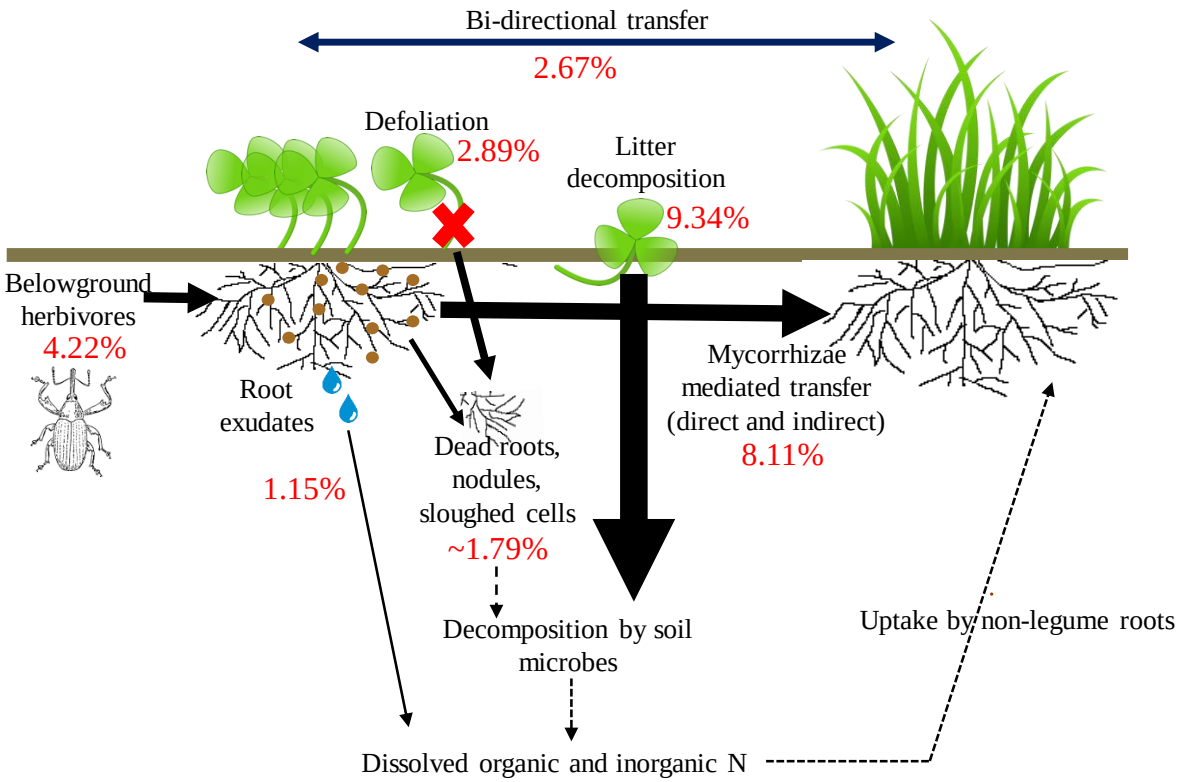


Figure 7.1. Main findings on the relevance and size of different N-transfer pathways, values in red represent $Ndft_R$ (proportion of non-legume N derived from the transfer of legume root N). $Ndft_R$ value for N-transfer with full interaction (no treatment) is the average of all comparable treatments conducted, it is assumed therefore, that within this treatment that decomposition of dead root and nodules would contribute towards two-thirds of total transfer (1.79%).

This work represents the first time that the biomolecular fate of N released from clover has been traced into the total hydrolysable AA or soil protein pool, either from the direct exudation of compounds or through microbial-mediated transformations (Figure 7.2). All experiments showed an overall low percentage of ^{15}N incorporation into the soil AAs, enforcing the requirement to achieve high ^{15}N enrichment when developing ^{15}N labelling methods. For the majority of treatments applied, preferential routing of ^{15}N was found into Glx, which correlated well with the literature in terms of N assimilation biochemistry (Section 1.3.1) and the fact that Glx was seen to be exudated in the greatest amounts with $CO(NH_2)_2$ application (Section 3.4.6.4 and 4.4.6). Further to this, the preferential routing and biosynthetic proximity of other AAs to Glx could be seen, with the incorporation into Hyp, Phe, Lys and Tyr always being seen to be lower than the other AAs. Results also enabled any potential effects (positive or negative) of different treatments on the routing of N and/or the soil microbial community to be identified, e.g. the clover cut treatment resulted in the highest incorporation of the applied label

into Ala, as well as seeing a significant reduction in the concentration of all individual AAs, therefore, potentially reflecting changes in microbial structure and nutrient cycling which have been seen as a result of defoliation. Therefore, a compound-specific approach may enable not only the cycling of compounds to be studied but allow indication of plant and soil health. This may be possible seeing as significant alterations in the AA concentrations within the soil were seen as a result of defoliation (with similar results being obtained through leaf application of ^{15}N enrichment compounds), possibly indicating plant stress. It has already been shown that plant composition can affect soil fauna functional groups and diversity (D'Annibale et al., 2017), therefore, it is likely that the specific management system would also have an effect.

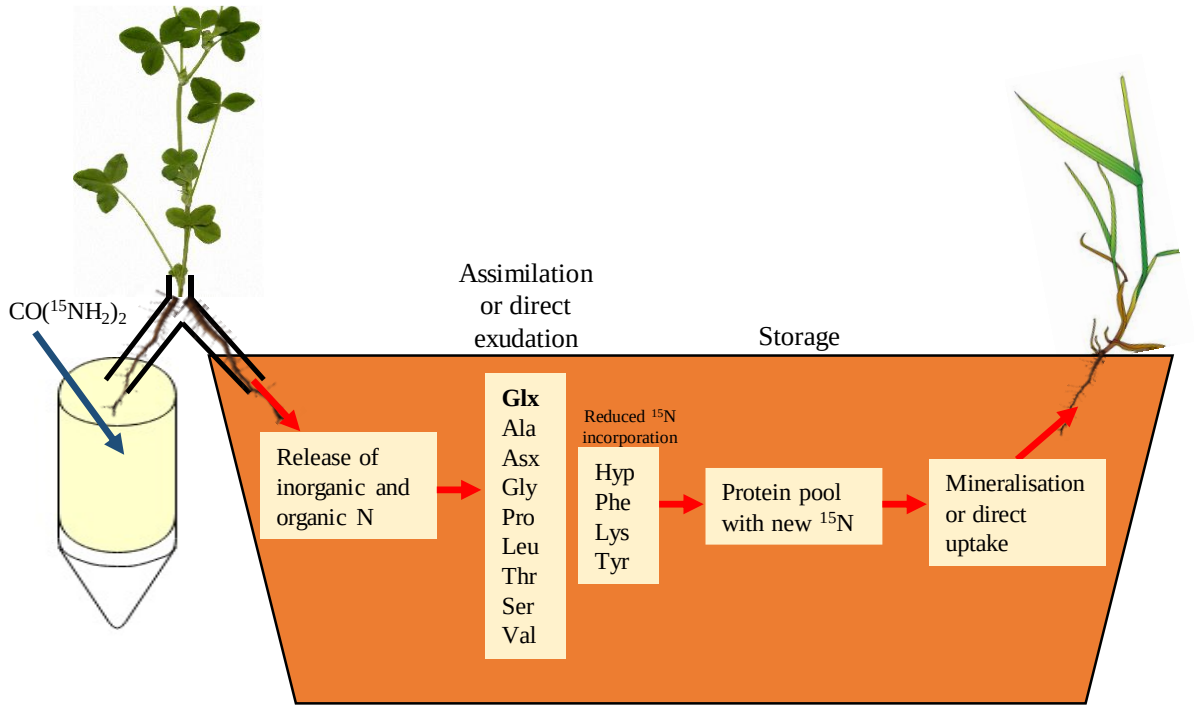


Figure 7.2. Diagrammatic summary of methodological approach and findings relating to soil AAs.

In terms of developing land-use management strategies, the evidence is compelling that the incorporation of clover shoot residues can provide maximal N-transfer, especially in the short-term. Furthermore, to provide a source of N in the longer-term whole crop residues could be incorporated to benefit from the slower decomposition of root material, this is overall most likely to be the most cost efficient and sustainable N source. It is known that decomposition processes are slow and that mineralisation is typically constant between years, so this is likely

to provide a constant N source with reduced variability that is experienced relying on N_2-fixation alone, which varies over growing seasons (Legdard, 2001). N-transfer between plants could further be maximised if necessary with the addition of AM fungi, although, consideration of a number of different factors is required first, including; relating to the inoculum (effectiveness, pathogen-free, shelf-life before application), the effects on other soil organisms and soil functioning alongside the economic cost of application and commercial availability (Jarstfer and Sylvia, 1993). Therefore, careful consideration is needed to ensure that any application is sustainable.

The work presented in this thesis has addressed the five specific objectives detailed in Section 1.5, contributing to greater scientific understanding of N-transfer between plants in agricultural grasslands, which could ultimately be used to develop land-use management strategies. A key achievement from this research was the development of a robust repeatable method which enabled easy manipulation and the investigation of a range of different treatments on N-transfer from clover-to-ryegrass, as well as using a novel compound-specific AA isotope technique to investigate the routing and microbial assimilation in the soil organic N pool. This approach enabled new insights to be gained into the molecular level processing of N in soils, questioning the use of the commonly used leaf-labelling technique and the effects of defoliation on N-cycling and ecosystem functioning. The results generated from studying different N-transfer pathways revealed the importance of decomposition in N-transfer and will be extremely useful in developing land-use management strategies aimed at improving the synchrony between clover and ryegrass, potentially increasing productivity and sustainability. This project is also valuable for directing further research based recommendations and large-scale land-use management strategies.

7.2. Future recommendations

Findings from this thesis have only just begun to understand the role of different pathways of N-transfer from clover to ryegrass, and still much remains unanswered about soil N cycling and the transfer of N between organic and inorganic soil N pools. In particular analysing inorganic N would allow greater insight into plant uptake, and transfer between different soil N pools. This could be done in a number of ways, but generally a diffusion method is used, trapping inorganic N on acidified filter disks before IRMS, this is a several step process where: NO_3^- is reduced to NH_4^+, then volatilized from the solution following the addition of MgO, before being captured on acidified glass fibre filters (diffusion of ammonia technique) (Bremner and Keeney, 1964; Stark and Hart, 1996; Sebilo et al., 2004; Chen and Dittert, 2008; Torres-Cañabate et al., 2008), although other methods do exist (Lachouani et al., 2010). This could be used to address possible experiment expansions and answer some of the questions raised throughout this project (Section 7.2.1), but also could be used to analyse soil material already collected from this project.

However, a major question that remains unanswered is: how N-transfer processes vary in the field, especially considering that on grazed grassland the input of excreta is likely to be a major pathway for N-transfer (Whitehead, 1970; Ledgard, 2001). There is also a need to establish the agricultural significance of N-transfer in natural environments (Giller et al., 1991) and ensure that N-transfer does not contribute to weed problems and reduced yield (Moyer-Henry et al., 2006). It is important to establish how the small laboratory incubations carried out relate and represent natural processes, thus determining the value of the experiments carried out. Therefore, it is necessary to adapt the split-root labelling technique for the field, using impermeable barriers to separate roots, then applying a similar range of treatments as within this project. In the field, resolving the incorporation of clover residues into the soil AA pool could be made possible by placing mesh bags filled with fresh clover material (0.2 × 1mm mesh) in ryegrass strands (Edmonds, 1980; Müller et al., 1988; Müller and Sundman, 1988; Breland, 1994a, b).

7.2.1. Further questions and extensions to existing experiments carried out in this thesis

7.2.1.1. Chapter 3: Developing a method for the application of a ^{15}N-label into white clover (*Trifolium repens*) to study nitrogen transfer

The preliminary chapter of this thesis focused on developing a method for investigating the uptake of a ^{15}N-label into a singular white clover plant, allowing the establishment of the best method for application allowing the aims of the study to be addressed. However, there are opportunities to extend these experiments and address different questions or areas of research, e.g. examining the different N uptake abilities of clover and ryegrass. Additional questions which could be investigated are:

(i) Does the uptake pattern of ^{15}N labelled compounds vary between clover and ryegrass (as well as between different species) with time?

(ii) How does the uptake of different N compounds (e.g. NH_4NO_3, NH_4Cl, KNO_3, $CO(NH_2)_2$, Glu, Asp) vary over time?

(iii) How long do elevated levels of ^{15}N remain in clover roots and shoots?

(iv) Can the same overall ^{15}N enrichment of clover plant parts be achieved with multi applications of $CO(NH_2)_2$ at a lower concentration and atom %?

(v) How can findings be used to up-scale experiments (i.e. larger volume of soil, longer duration experiments)?

(vi) What proportion of the ^{15}N applied is not taken up by clover roots?

(vii) What is the best way to simultaneously measure the amount of N_2-fixed by clover and the amount of N-transferred?

(viii) What is the significance of the change in exudation pattern with uptake of different N sources? E.g. plant uptake of different N sources effects N-cycling and root exudates stimulate root nodulation and N_2-fixation (Coskun et al., 2017), therefore what are the wider implications?

Questions (i-iv) could be addressed through the adaptation of the methods used in Chapter 3, using a split-root labelling technique sampling over-time (i.e. 1, 2, 3, 6, 24, 30, 54, 100, 173, 361 hours) and further extending the experiment to see how ^{15}N enrichment changes over longer time periods [for example, up to 3 months (2190 days)]. Question (v) could be addressed by using larger incubation tubes, hence larger volumes of soil. By addressing these questions,

question (vi) could be investigated in the LC. Question (vii) could be resolved by growing legume and non-legume plants in ^{15}N enriched soil, with measurements of N_2-fixation made based on the dilution of ^{15}N in the non-legume plant, using further incubations to act as reference plants (Hardarson and Danso, 1993). Investigating this would then allow question (viii) to be addressed, where N_2-fixation could be measured with the application of different ^{15}N enriched fertilisers, alongside assessing nodulation.

7.2.1.2. Chapter 4: Investigation of the routing and controls of nitrogen transfer between clover and ryegrass

Chapter 4 looked at building on the methods developed in Chapter 3 to investigate quantifying the amount of N-transferred between clover and ryegrass and vice-versa, finding that N-transfer was seen to be equal in both directions. Therefore, it is important to see how this is affected under N limiting conditions and under what conditions ryegrass becomes more reliant on N released from clover. Additional questions which could be investigated are:

(i) How does the incorporation of ^{15}N into soil AAs vary over-time? How long does the ^{15}N-label remain present in the soil AA pool?

(ii) When ryegrass uptake of soil ^{15}N is low, is the additional input by clover into the soil N pool prone to leaching and loss?

(iii) How does the amount of N-transferred between clover and ryegrass and vice-versa vary with soil N content? How do other soil properties (soil type, clay content, pH, land management history etc) effect the amount of N-transferred?

(iv) How does the amount of N-transferred vary with different ratios of plants (i.e. 2 clovers:1 ryegrass, 1 clover: 2 ryegrass)?

(v) What is the inorganic N component in plant exudates? How does inorganic and organic N content of exudates vary between clover and ryegrass?

(vi) How does N-transfer differ with removal of root contact and intermingling roots?

(vii) In what chemical form(s) is ^{15}N taken up from soil into clover and ryegrass, and does this vary when different substrates are initially applied in the LC? Does ryegrass have a preference for inorganic or organic N uptake?

(viii) How much of the soil ^{15}N incorporated into the soil is within the soil inorganic N pool?

(ix) How does the inorganic N component of exudates relate to the incorporation into soil AAs and transfer from inorganic to organic pool and vice-versa? What proportion of AAs in exudates and in soil are 'free' AAs?

(x) What is the routing into other soil organic N components?

Questions (i-vii) could be addressed through the adaptation of methods used within Chapter 4. For example, Question (ii) through repeating incubations and investigating leaching by flushing N soluble compounds from the soil. Question (iii) could be addressed by using a variety of different soils, collecting soils from a range of agricultural settings and repeating experiments, with possibilities for addressing question (vi) being illustrated in Figure 7.4. Samples collected throughout this project could be used to address question (vii) (Section 7.2). Addressing questions (v) and (viii), will ultimately allow question (ix) to be addressed, alongside AA extraction of soil and exudate samples without acid hydrolysis (Section 2.4.4) allowing free AAs to be quantified. While question (x) would require significant analytical method development as only a small portion of organic N is currently identified, therefore, the next logical challenge would be the routing into amino sugars which make up approximately 5-6% of total soil N (Schulten and Schnitzer, 1998).

Although not related to the aims of the study, one of the interesting findings from Chapter 4 was the effect that the leaf-labelling technique had on the soil AA concentrations, which warrants further investigation for this commonly used technique to establish whether this finding is universal. As an initial starting point, leaves from a range of different plant species could be immerged in ^{15}N enriched compounds (e.g. NH_4NO_3, NH_4Cl, KNO_3, $CO(NH_2)_2$, Glu, Asp) and soil AAs monitored as well as plant tissue AAs.

7.2.1.3. Chapter 5: Investigation of the role of exudation and decomposition in nitrogen transfer

Chapter 5 looked at a handful of different exudation and decomposition processes which could be manipulated using the developed method, however, this was limited by the number of different treatments which could be maintained at the same time. There are a number of other scenarios which could be investigated using the same experimental set up, which are illustrated in Figure 7.3.

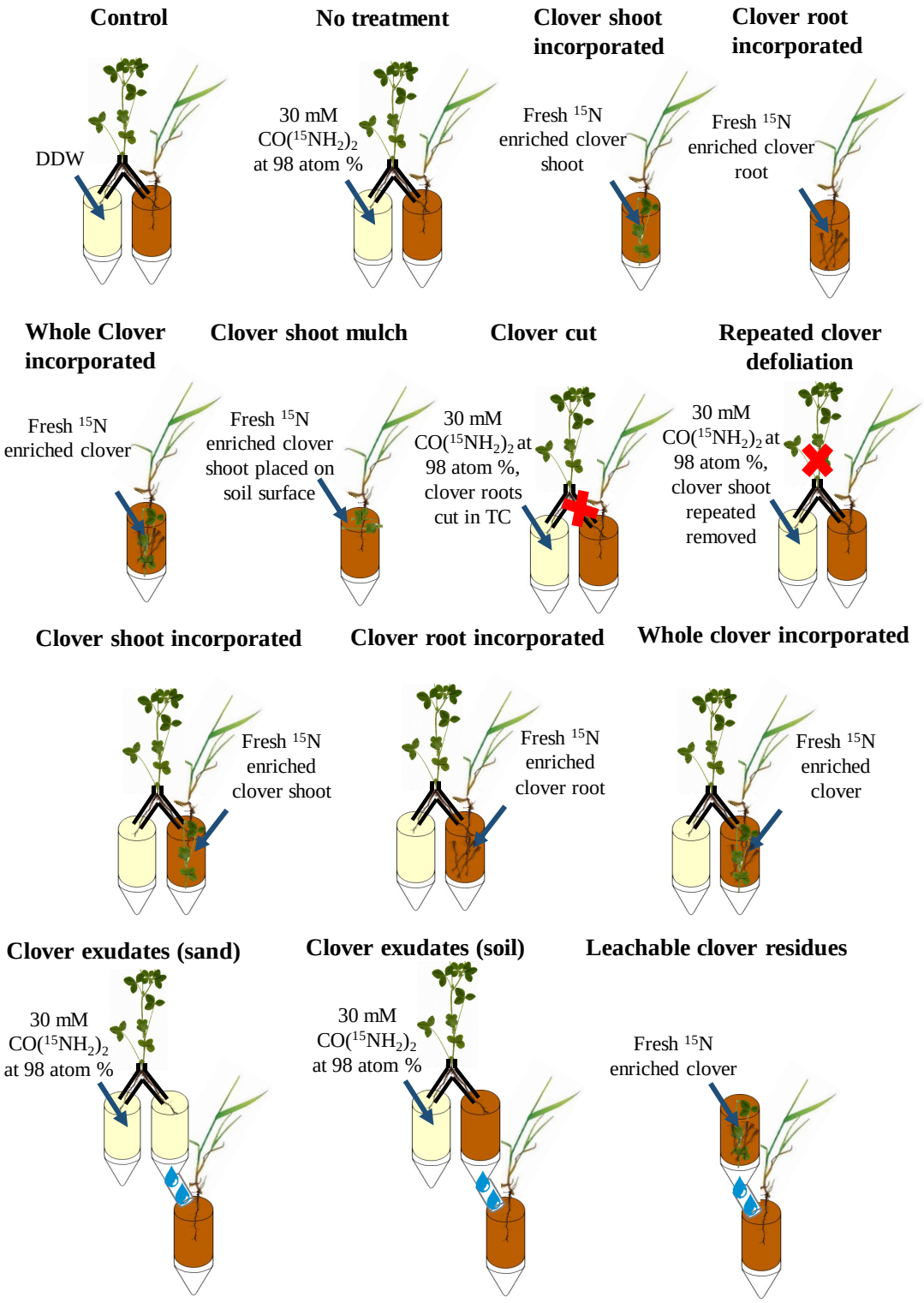


Figure 7.3. Proposed expansion of experiments conducted within Chapter 5, allowing the N-transfer pathways to be further studied.

Further to Figure 7.3, additional questions which could be investigated are:

(i) How do different N-transfer pathways vary over-time? Does exudation play a greater role in the first few hours or days after ^{15}N labelling compared to after weeks?

(ii) Can the incorporation of clover root and shoot material provide a longer-term N supply to an associated crop?

(iii) How does N-transfer differ with removal of root contact?

(iv) How does repeated defoliation, cattle grazing and excreta inputs effect the amount of N-transferred and the transfer pathway?

(v) What effect does the incorporation of clover residues have on soil based food webs and functional diversity of soil organisms?

(vi) How does decomposition of clover residues vary with pervious land use? Is the home-field advantage (HFA) hypothesis important in determining N-transfer pathway?

Questions (i-iv) could be addressed through the adaptation of methods used within Chapter 5. For example, question (i) could be addressed using a similar temporal study as in Chapter 3 (1, 2, 3, 6, 24, 30, 54, 100, 173, 361 hours) capturing events in the short-term as well as in the longer-term with the different treatments studied in Chapter 5. Further extending the study would allow N-transfer over months (instead of weeks) to be investigated, Question (ii) could then be addressed using whole clover residues. Question (iii) could similarly be addressed using the treatments in Chapter 5 with adaptation of the method as shown in Figure 7.4. Question (iv) could be addressed by expanding the proposed extensions to experiments in Figure 7.3 to look at other N-transfer pathways. To address question (v) several different methods could be used, including; microbial respiration, C source utilisation (using Biolog plates with 96 different C sources), isolating microbial DNA to monitor changes in community structure (use of polymerase chain reaction), bacterial plate counts, population counts (microarthropods, nematodes) (Wardle et al., 1995; Ölinger et al.,1996; Derry et al., 1998; Clayton et al., 2005).

An important point raised in Chapter 5 [question (vi)] was whether microbes become tuned to receiving a certain type of plant litter (i.e. HFA), this requires further investigation especially if agriculture is to move towards increasing reliance on BNF and crop associations with legumes, where decomposition plays a major part in N-transfer between legumes and non-legumes. This area of research is important in the conversion to arable pastures and within crop rotations to develop appropriate land-use management strategies. Therefore, it is desirable to

study N-transfer with a range of different soil types and land uses (which could easily be done using the methods set out in this thesis), as well as different legume and non-legume associations (e.g. soya bean and maize, pea and barley, lupin and rapeseed).

7.2.1.4. Chapter 6: Investigation of the role of soil biota in nitrogen transfer from clover to ryegrass

Chapter 6 looked at a handful of manipulations or additions to the soil biota community which are thought to play a fundamental role in N-transfer, however, this was limited by the number of different treatments which could be maintained at the same time. Therefore, similarly to detailed in Section 7.2.1.5 this could be expanded upon, looking at a whole range of different organisms, e.g. nematodes, collembola, earthworms [which have been shown to significantly increase N-transfer between plants (Schmidt and Curry, 1999)]. However, Chapter 6 also detailed the ambiguous results for direct N-transfer through CMNs, which warrants further investigation. The current experimental design would not permit this to be investigated due to the small incubation tubes, therefore, these would need to be upscaled, allowing an experimental design as illustrated in Figure 7.4 to be conducted. This would allow the contribution of CMNs to N-transfer to be quantified linking to results from Chapter 5. It is therefore necessary in expanding this experiment to look at the mycorrhizal status of plants and the extent to which roots are colonised with mycorrhizal fungi. The percentage of root colonisation is typically obtained by the gridline intersect method (Hamel et al., 1991b; Frey and Schüepp, 1992) where the root system is spread out and the presence or absence of infection is recorded (Giovanetti and Mosse, 1980).

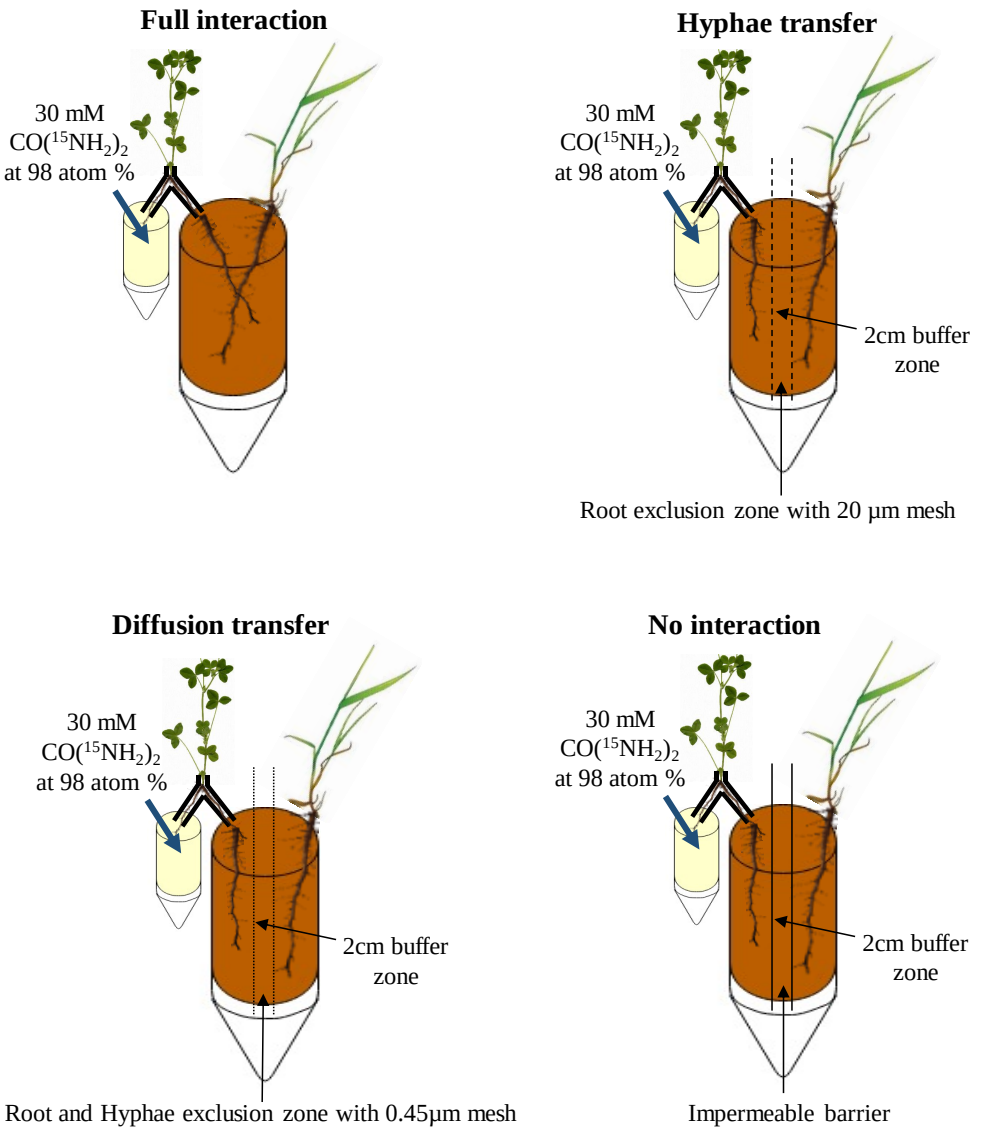


Figure 7.4. Future work on establishing the role of mycorrhizal fungi in direct N-transfer using different mesh sizes to control the interaction level between clover and ryegrass plants. A large TC would be required to allow a mesh to be inserted to separate the roots. The buffer zone allows transfer via mycorrhizal fungi to be detected more easily. Method adapted from: Frey and Schüepp, 1992; Jensen, 1996b; Johansen and Jensen, 1996; Tannin et al., 2000.

Further to Figure 7.4, additional questions which could be investigated are:

(i) Can N-transfer between clover and ryegrass be enhanced by selection of appropriate AM fungi species? Do AM fungi enable direct transfer of N between plants (Figure 7.4)?

(ii) Can the addition of a range of soil biota increase N-transfer between clover and ryegrass? What about the application of multi different soil biota?

(iii) How can different management techniques (i.e. clover incorporation) and application of different soil biota be manipulated to increase transfer, i.e. changing decomposition rates?

(iv) What effect do additions of different soil biota have on the functioning of soil process, microbial communities, functional groups and population numbers?

Questions (i-iii) could be addressed through the adaptation of methods carried out within Chapter 6. For example, question (ii) by repeating experiments using a range of different AM inoculum, question (ii) by using a range of soil biota. Question (iii) could be addressed by combining methods from Chapters 5 and 6. While question (iv) could use the methods outlined in Section 7.2.1.3, as well as a range of new and novel techniques, including the use of the detection and identification of active microbes responsible for the assimilation of ^{15}N through RNA and DNA, stable isotope probing (^{15}N-RNA-SIP, ^{15}N-DNA-SIP) (Buckley et al., 2007; Addison et al., 2010).

7.3. Concluding remarks

Overall, the work presented in this thesis develops a robust method, validates current opinions on the processes behind N-transfer, as well as demonstrating and applying a novel compound-specific AA isotope approach to investigating N cycling through plant-soil systems. This coupled with further research could allow N-transfer models to be developed, which would enable the 'black-box' on N-transfer to be fully resolved, predicting N availability to plants and allowing effective land-use management strategies across a variety of landscapes to be developed.

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Appendices

Appendices

Appendix A1

Biosynthesis pathways for AAs studied and quantified in this project

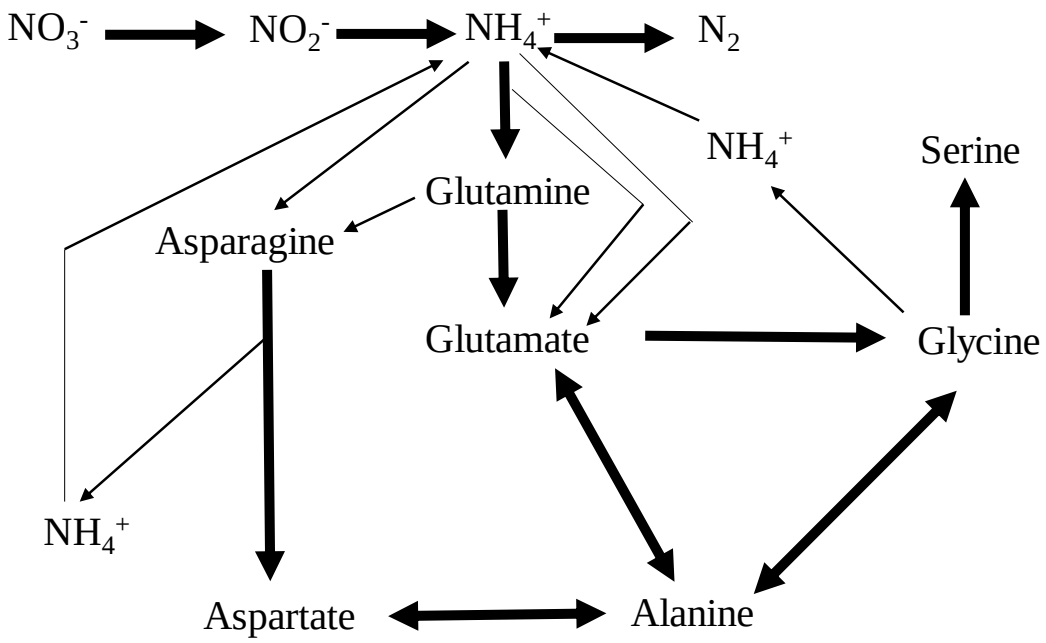

Figure A1.1. Main routes of nitrogen flow into amino acid biosynthesis (Adapted from Bowsher et al., 2008)

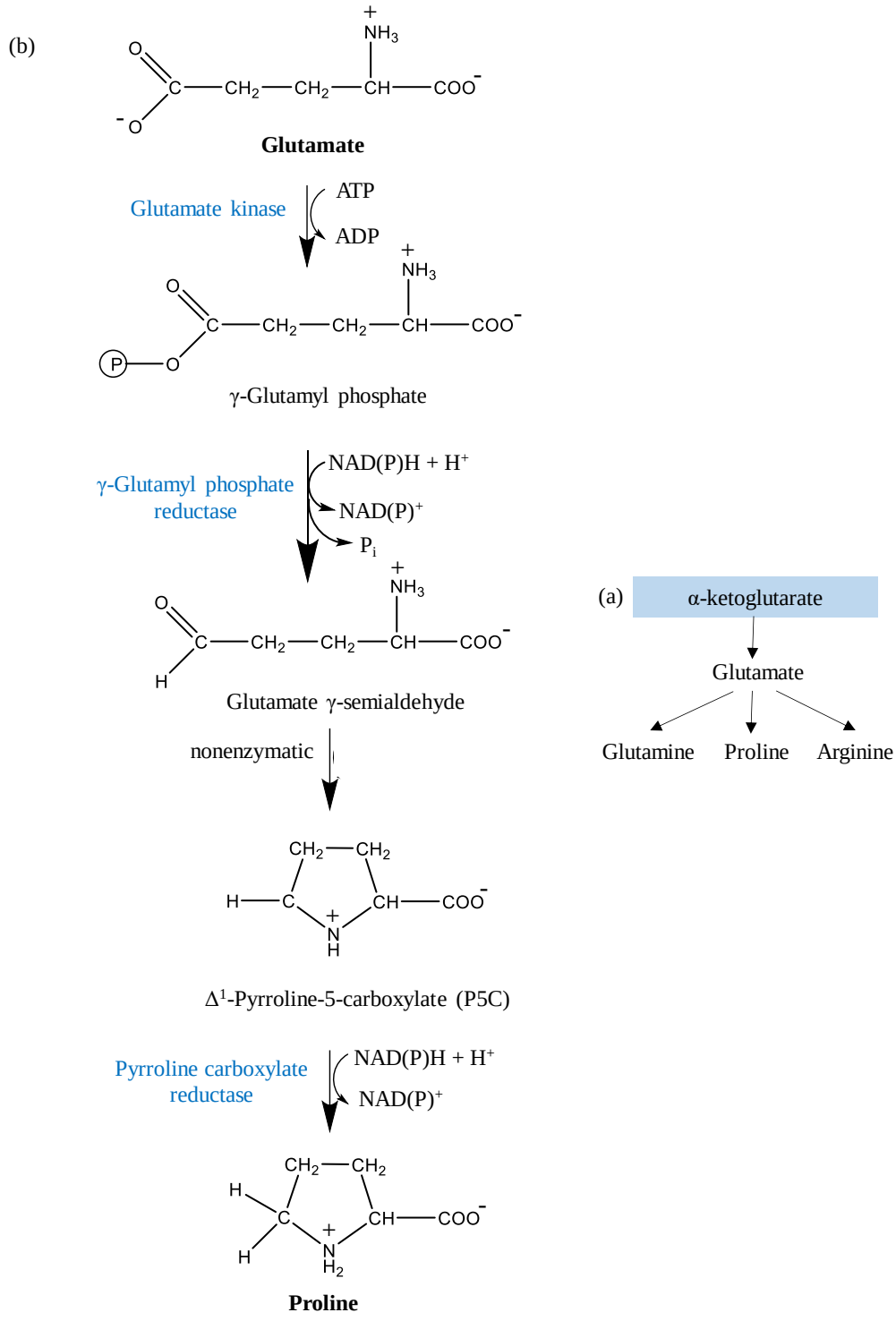

Figure A1.2. (a) Four AAs are derived from α-ketoglutarate, pathways for glutamate and glutamine are shown in Chapter 1 (Section 1.3.1). (b) Biosynthesis of Proline. (Adapted from Nelson and Cox, 2013)

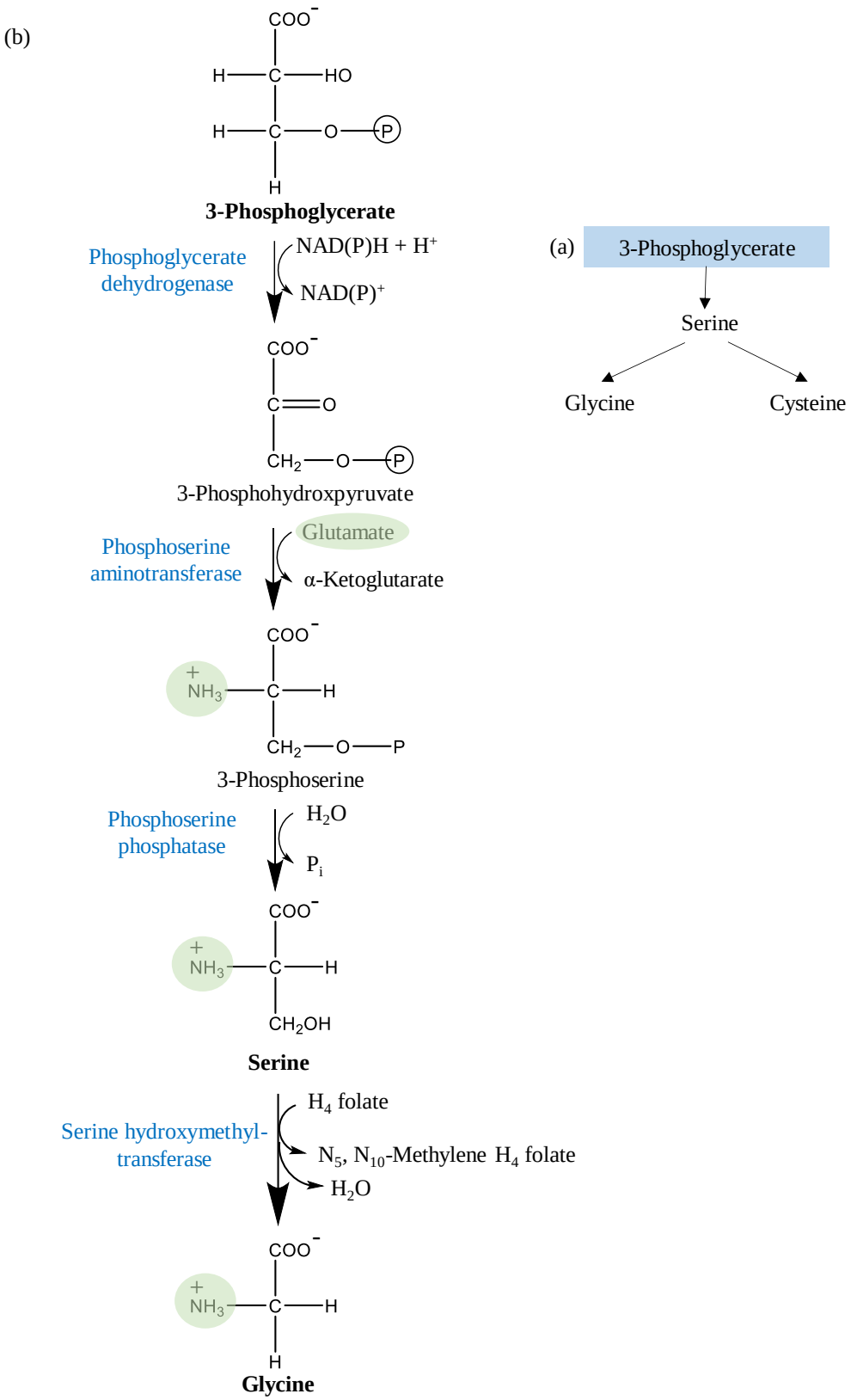

Figure A1.3. (a) Four AAs are derived from 3-phophoglycerate (with cysteine not being studied in this project). (b) Biosynthesis of serine and glycine. Green shading shows the transfer of an amino group (Adapted from Nelson and Cox, 2013.)

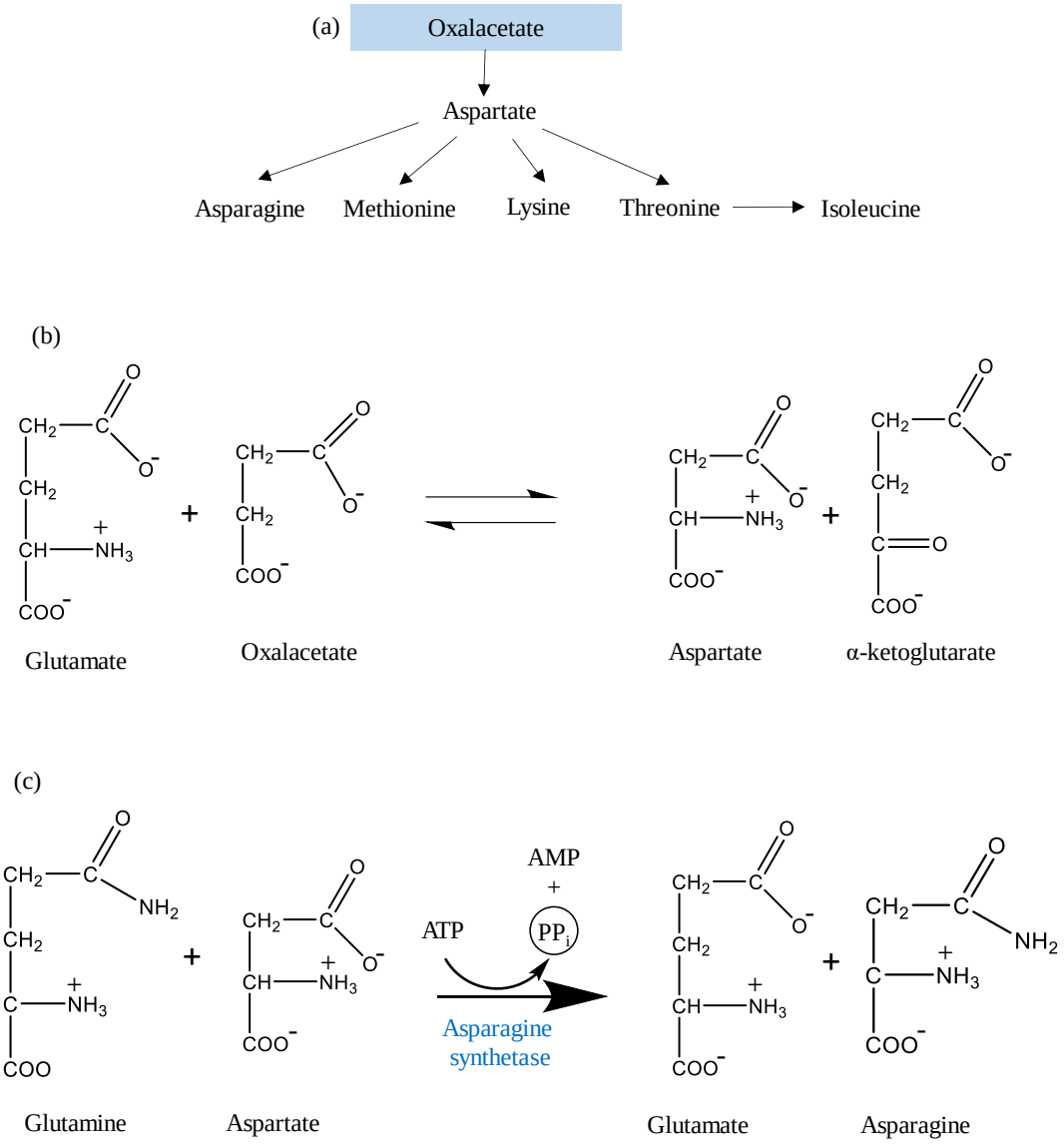

Figure A1.4. (a) Six AAs are derived from oxalacetate (with methionine and isoleucine not being studied in this project, Asp and Asn were converted to Asx under hydrolysis conditions). (b) Biosynthesis of aspartate. (c) Biosynthesis of asparagine from the conversion of aspartate and glutamine (Adapted from Bowsher et al., 2008; Nelson and Cox, 2013.)

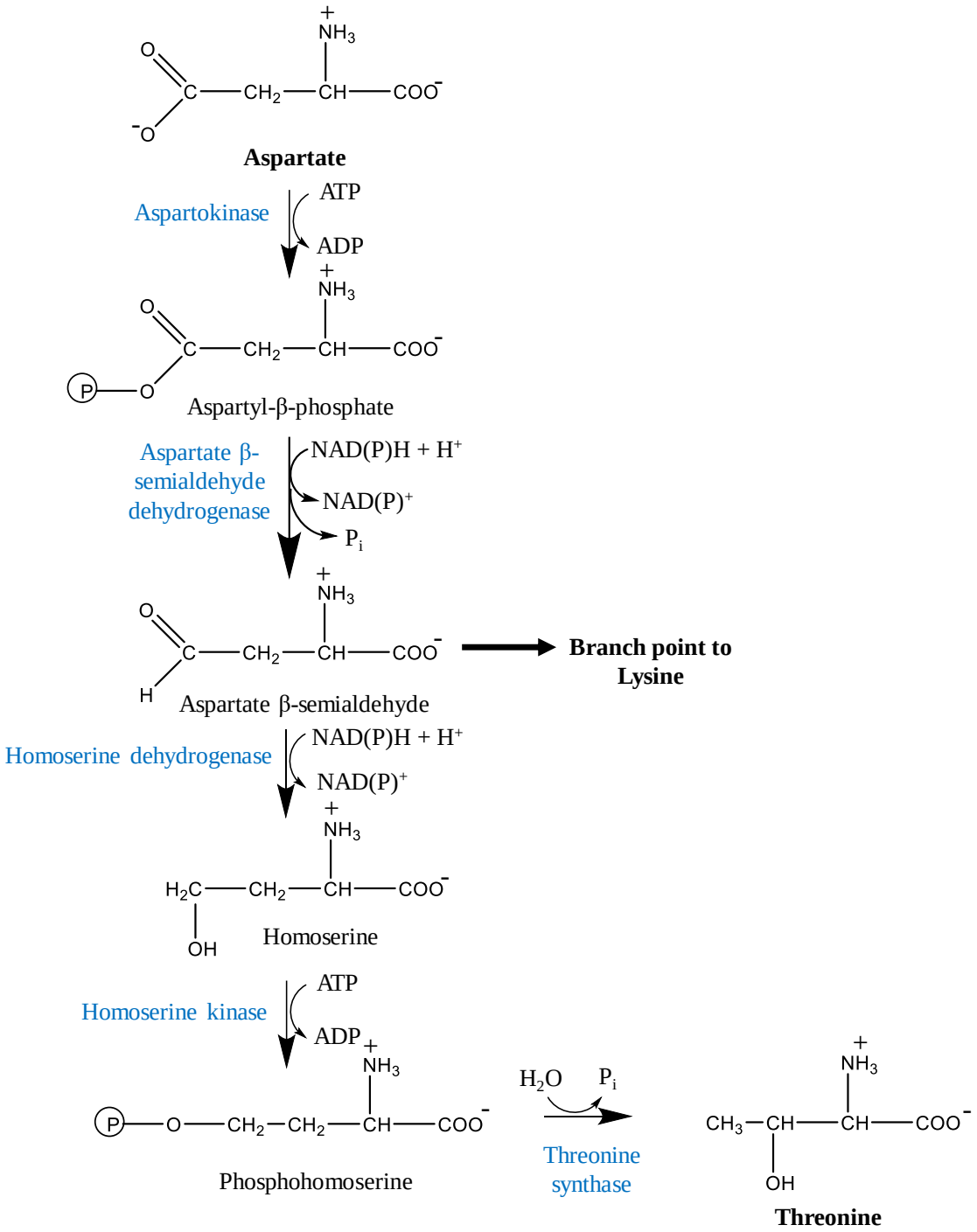

Figure A1.5. Biosynthesis of threonine, aspartate β-semialdehyde is the branch point for threonine or lysine synthesis (Adapted from Nelson and Cox, 2013.)

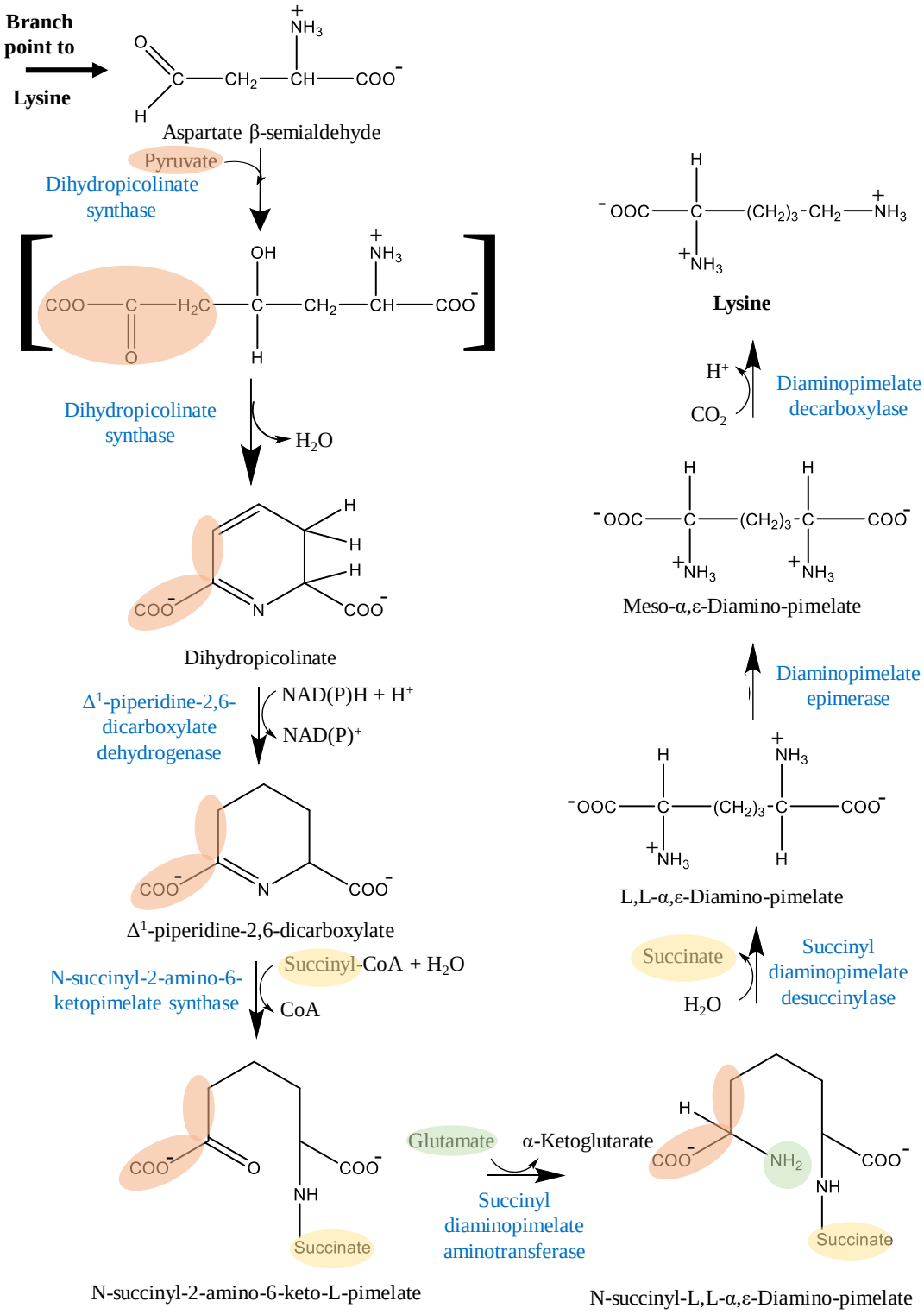

Figure A1.6. Biosynthesis of Lysine originated from aspartate which is shown in Figure A1.5, aspartate β-semialdehyde branch point either leads to threonine or lysine biosynthesis. Shading shows the transfer of different functional groups. (Adapted from Nelson and Cox, 2013.)

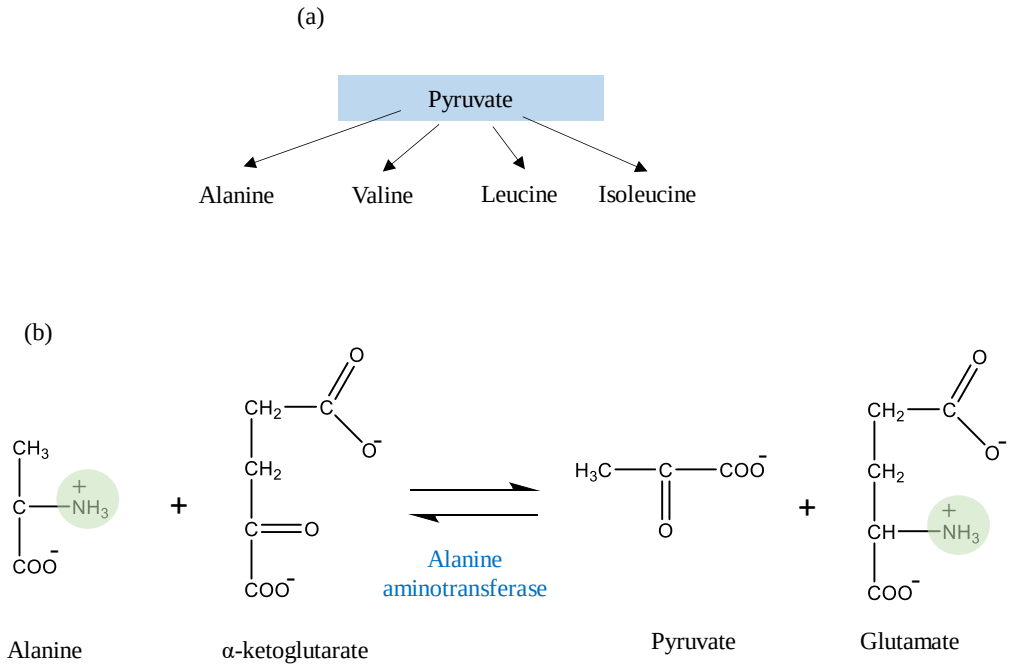

Figure A1.7. (a) Four AAs are derived from pyruvate (with isoleucine not being studied in this project). (b) Biosynthesis of alanine. Green shading shows the transfer of an amino group. (Adapted from Bowsher et al., 2008; Nelson and Cox, 2013.)

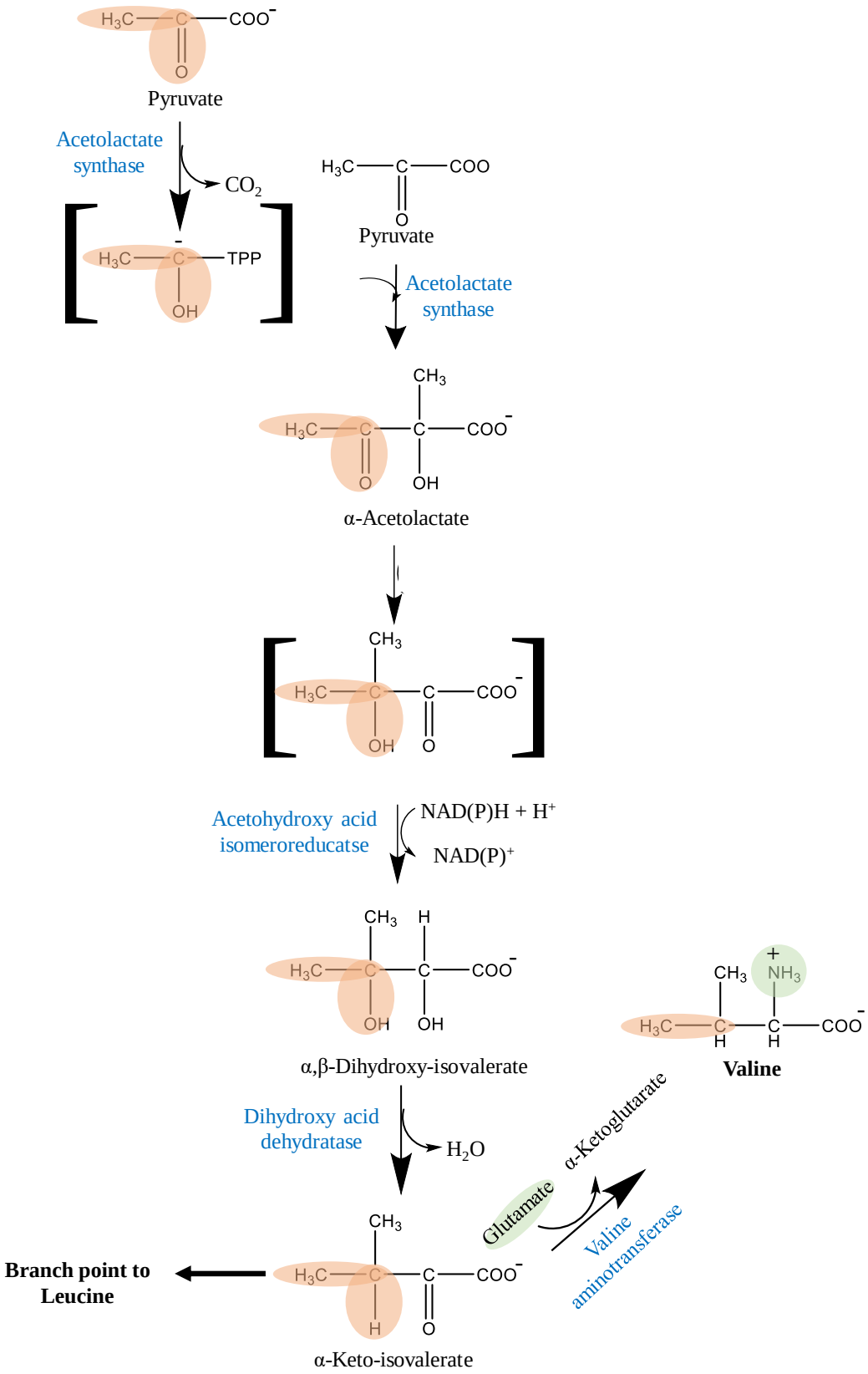

Figure A1.8. Biosynthesis of valine, α-keto-isovalerate is the branch point for valine or leucine synthesis. Shading shows the transfer of different functional groups. (Adapted from Nelson and Cox, 2013.)

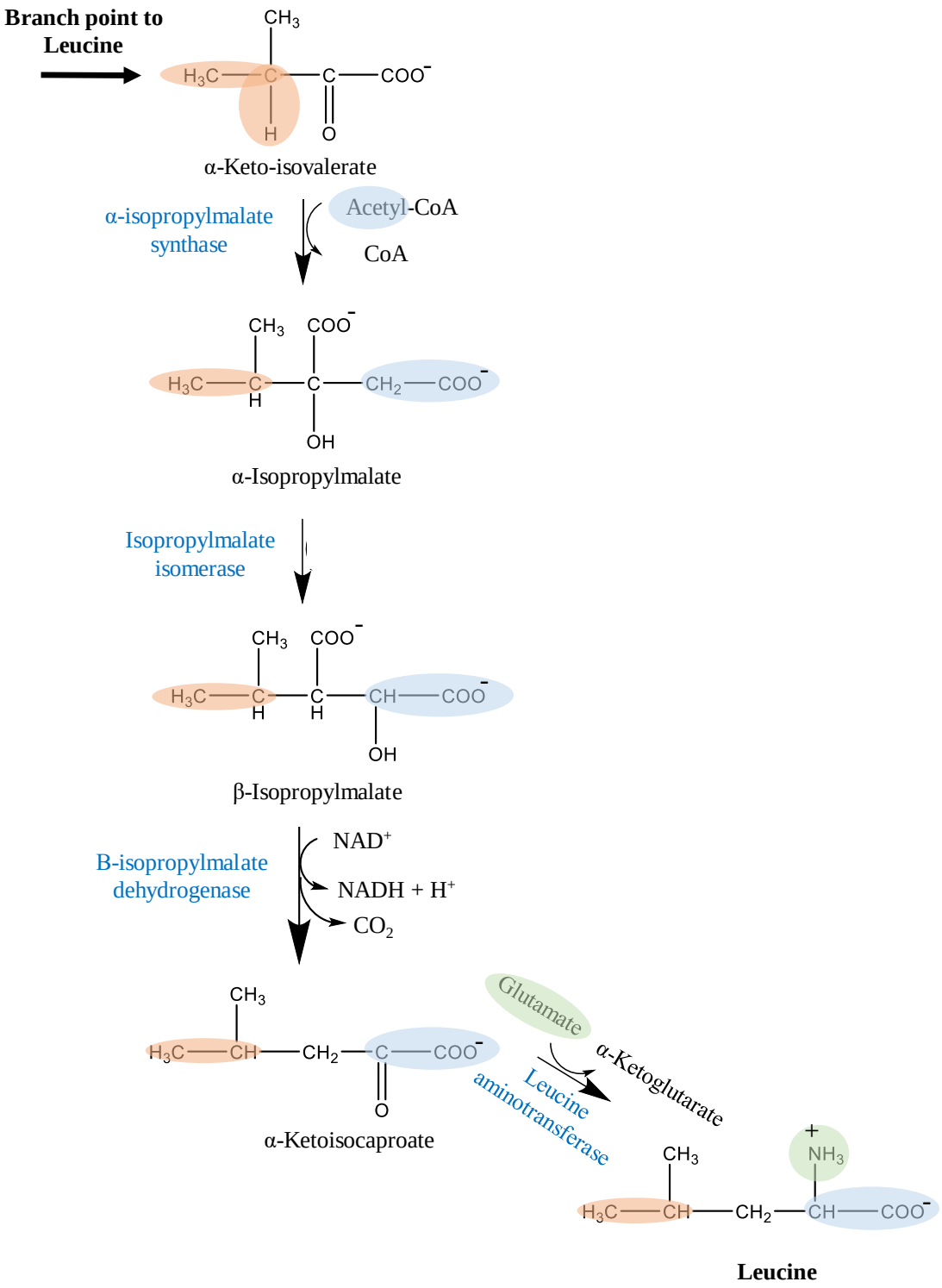

Figure A1.9. Biosynthesis of leucine originated from pyruvate which is shown in Figure A1.8, α-keto-isovalerate branch point either leads to valine of leucine biosynthesis. Shading shows the transfer of different functional groups. (Adapted from Nelson and Cox, 2013.)

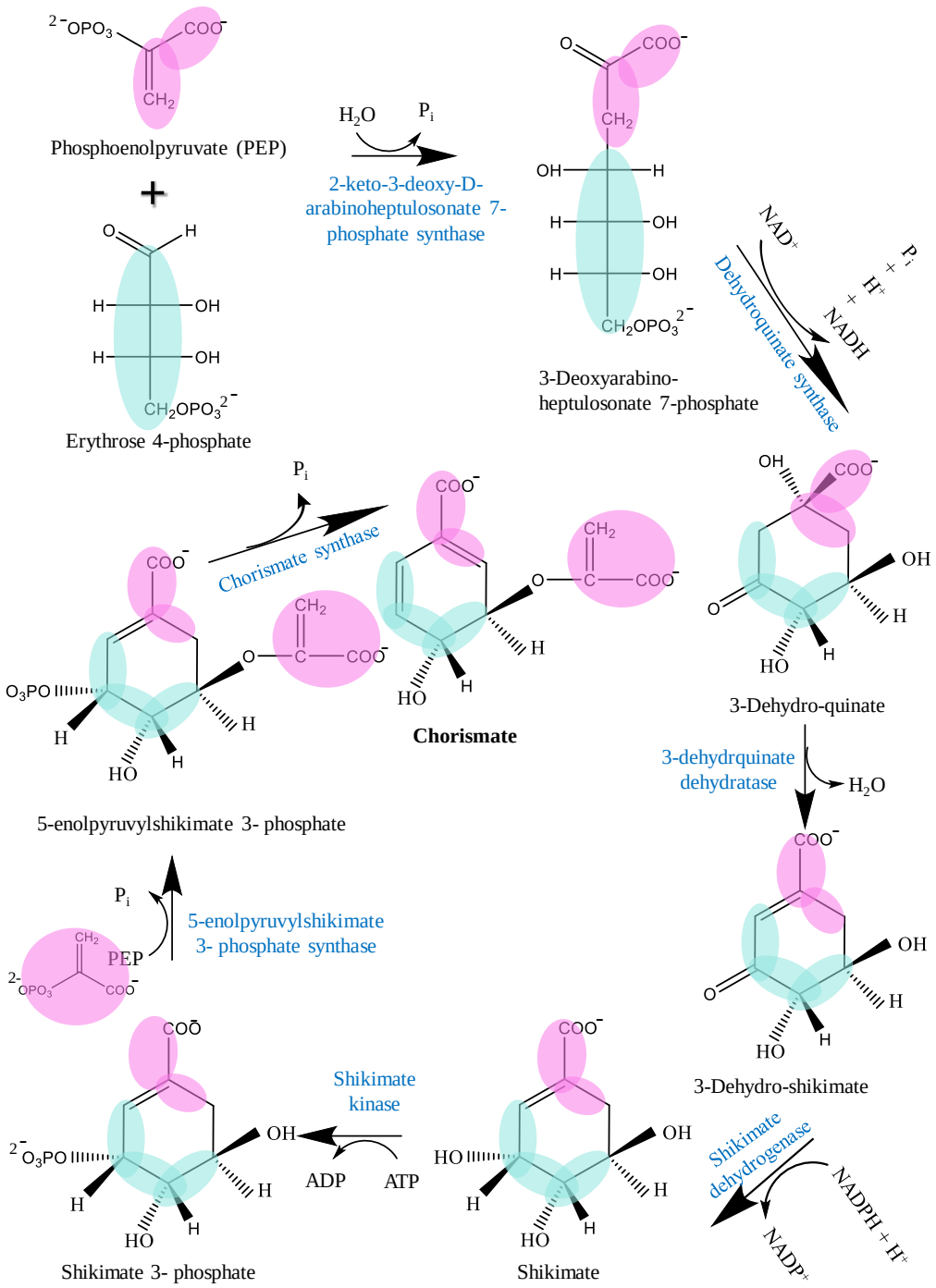

Figure A1.10. Biosynthesis of chorismate from phosphoenolpyruvate and erythrose 4-phosphate. Chorismate biosynthesis can then be use for the production of phenylamine and tyrosine (Figure A1.11). Shading shows the transfer of different functional groups. (Adapted from Nelson and Cox, 2013; Berg et al.2015.)

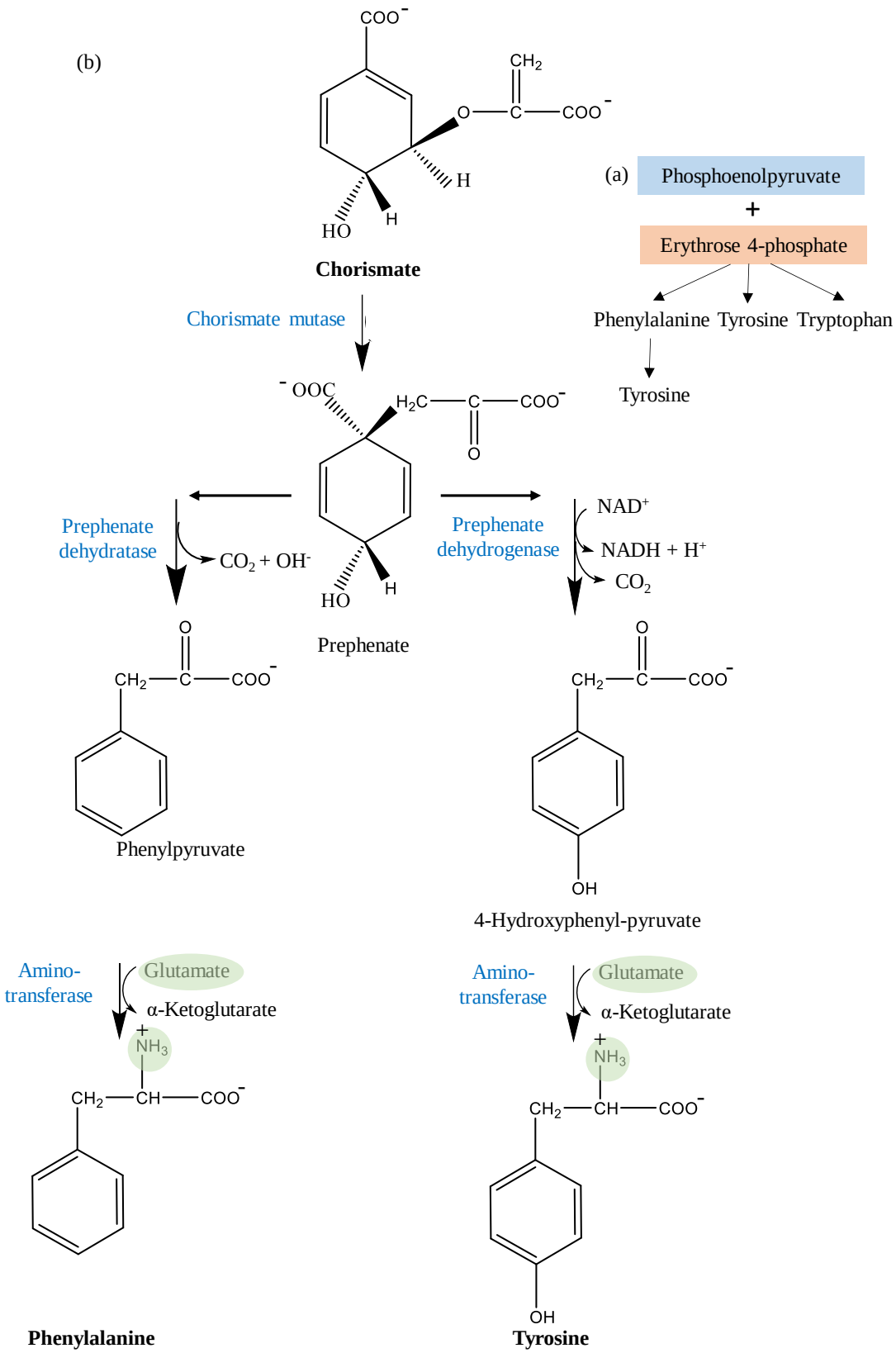

Figure A1.11. (a) Three AAs are derived from phosphoenolpyruvate and erythrose 4-phosphate (with tryptophan not being studied in this project). (b) Biosynthesis of phenylamine and tyrosine from chorismate biosynthesis (Figure A1.10). Shading shows the transfer of different functional groups. (Adapted from Nelson and Cox, 2013; Berg et al.2015.)

Appendix A2

Figures presented in thesis before outliers were removed

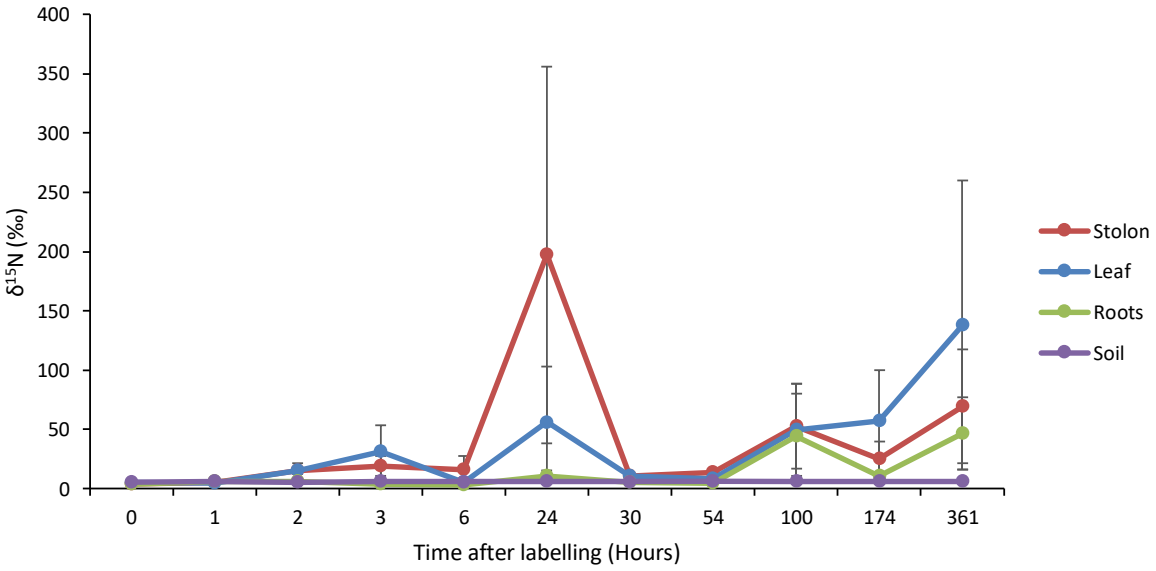

Figure A2.1. Temporal changes in $\delta^{15}N$ values for the leaves, stolon, roots of white clover (*Trifolium repens*) plants and soil growing in rhizotrons after leaf-labelling with 30 mM $^{15}NH_4{}^{15}NO_3$ at 10 atom % (without outliers removed, as in Figure 3.8). (mean ± standard error; n=4)

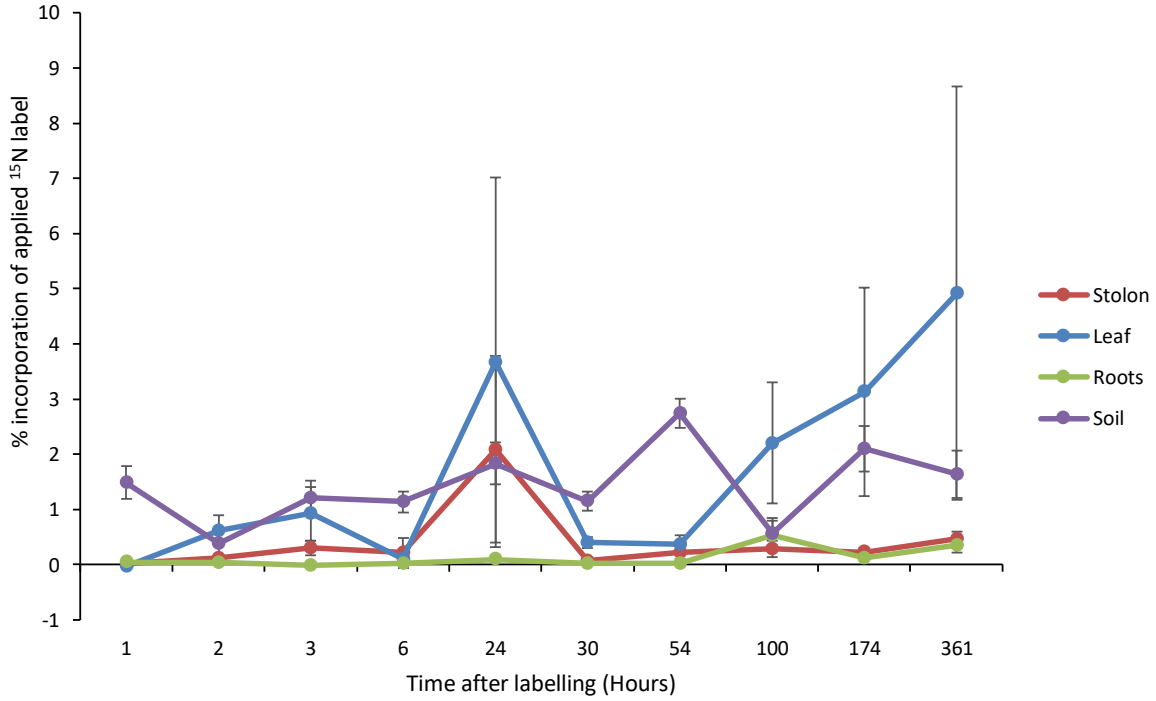

Figure A2.2. Percentage incorporation of the applied ^{15}N-label (30 mM $^{15}NH_4{}^{15}NO_3$ at 10 atom %) to white clover leaves (*Trifolium repens*) incorporated into different plant parts and the soil (without outliers removed, as in Figure 3.9). (mean ± standard error; n=4)

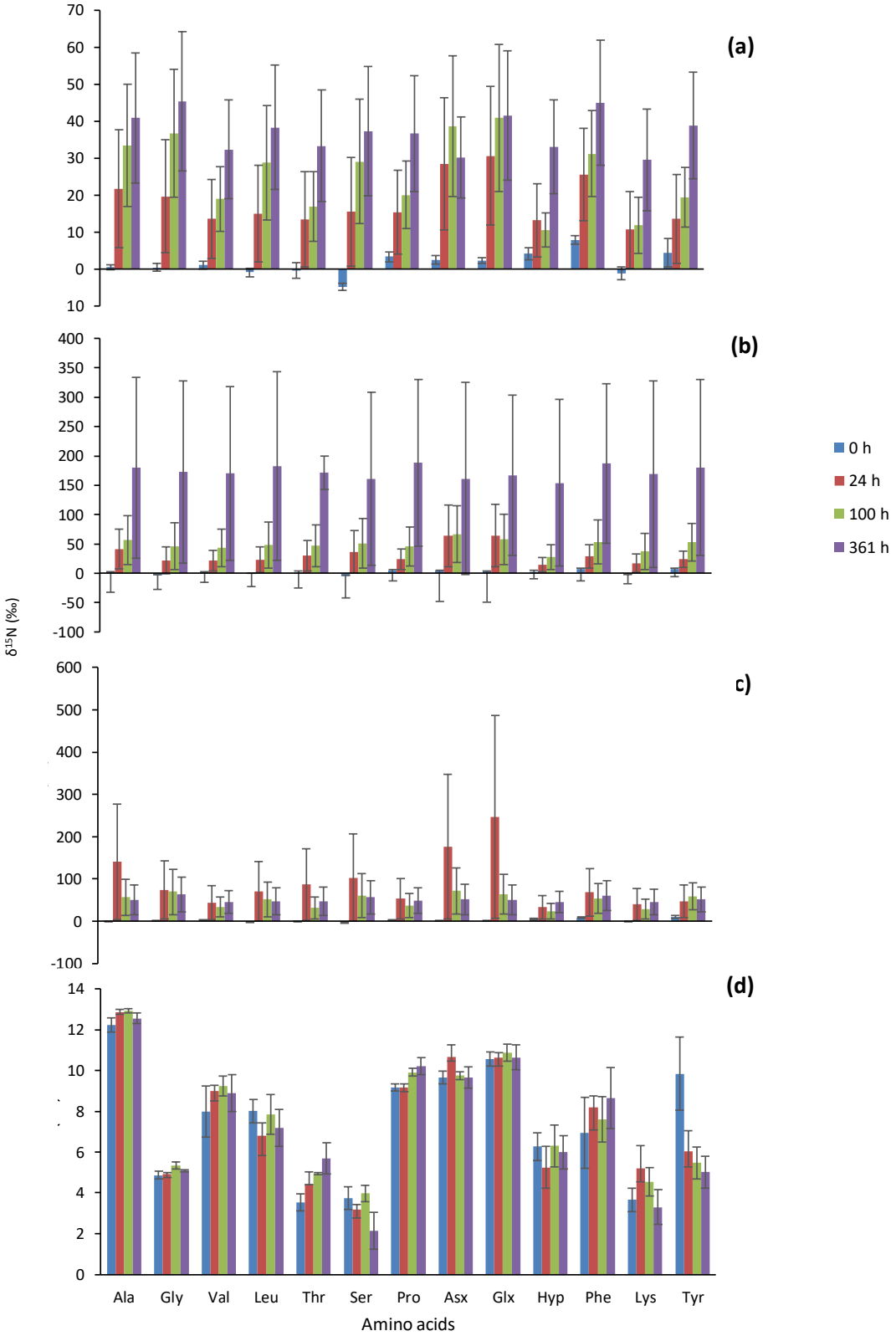

Figure A2.3. $\delta^{15}N$ values of the AAs in (a) stolon, (b) leaves, (c) roots and (d) soil of white clover (*Trifolium repens*) plants growing in rhizotrons following the application of $^{15}NH_4{}^{15}NO_3$ (30 mM at 10 atom %) through the leaf-labelling technique (mean ± standard error; n=3 4, without outliers removed as in Figure 3.11)

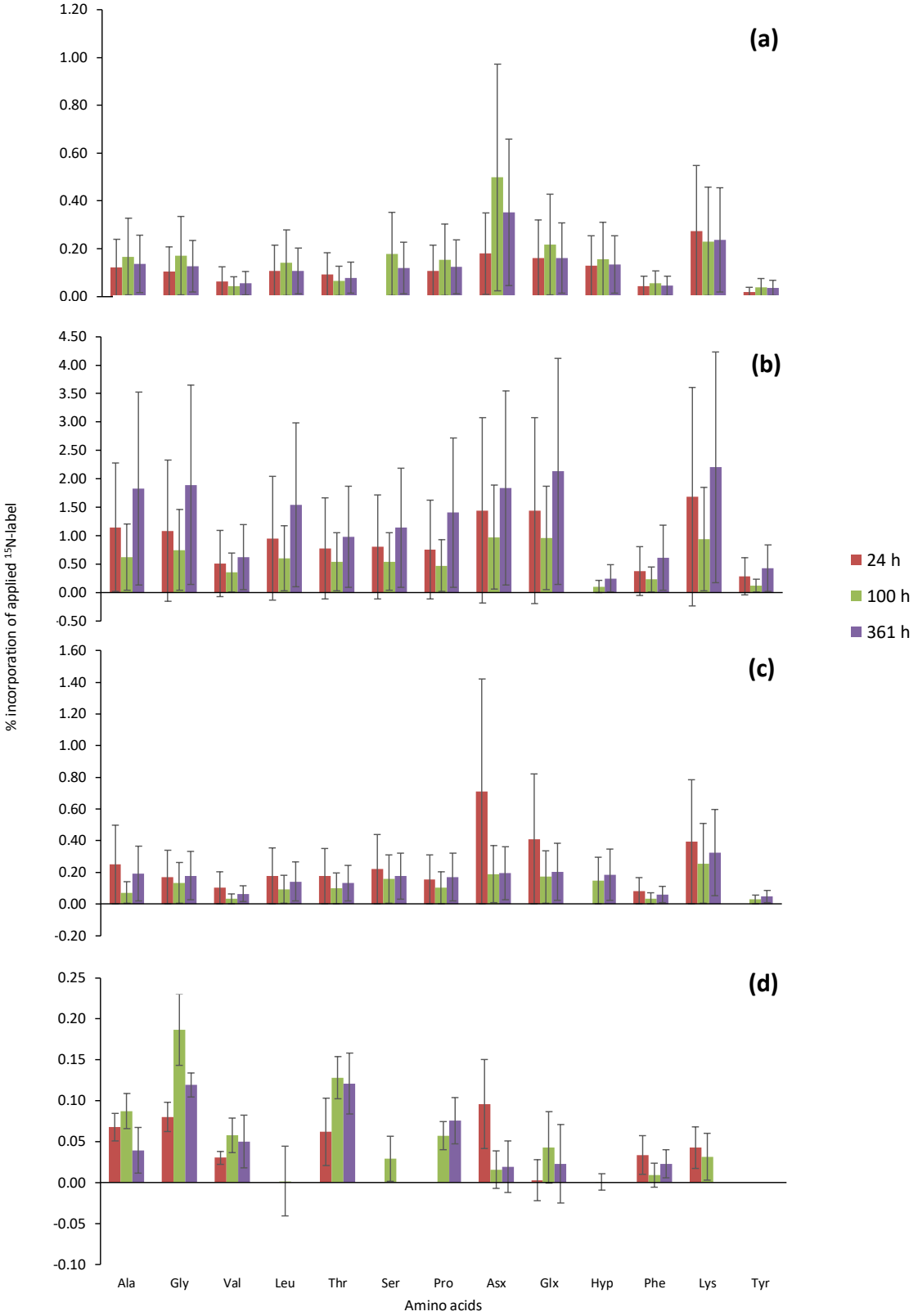

Figure A2.4. Incorporation of the applied ^{15}N-label (30 mM $^{15}NH_4{}^{15}NO_3$ at 10 atom %) through the leaf-labelling technique into individual AAs (%) in the different plant parts of white clover (*Trifolium repens*) (a) stolon, (b) leaves, (c) roots and (d) soil in rhizotrons over-time. (mean ± standard error; n=4, without outliers removed as in Figure 3.12.)

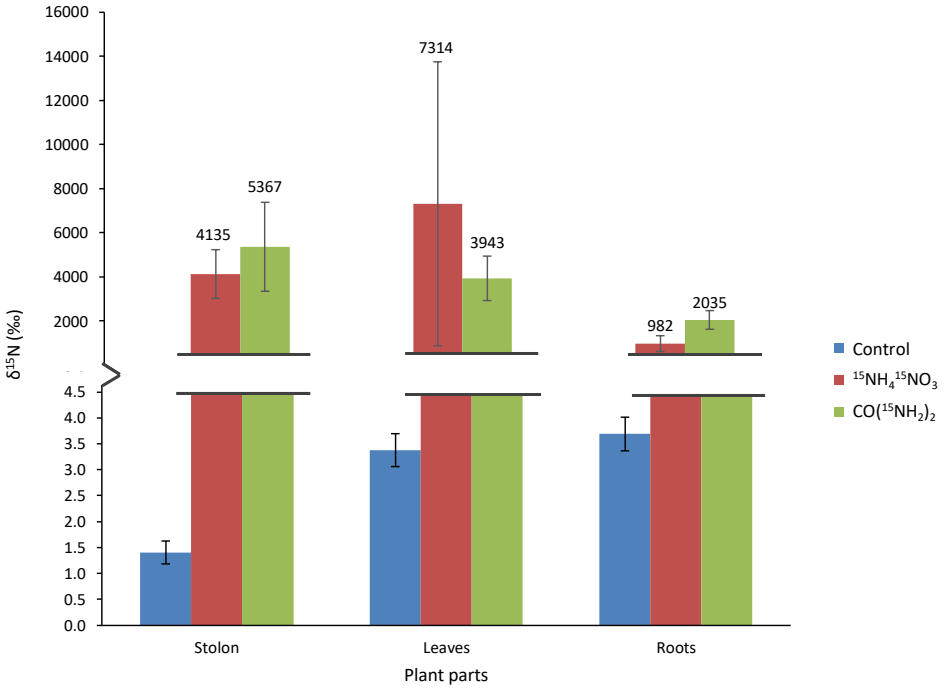

Figure A2.5. $\delta^{15}N$ values of different plant parts in each treatment for white clover (*Trifolium repens*) plants growing in sand within rhizotrons, plants either received DDW for the control, $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ (30mM at 98 atom %) through the leaf-labelling technique and sampled after 100 h. (mean ± standard error; n=6, without outliers removed as Figure 3.13.)

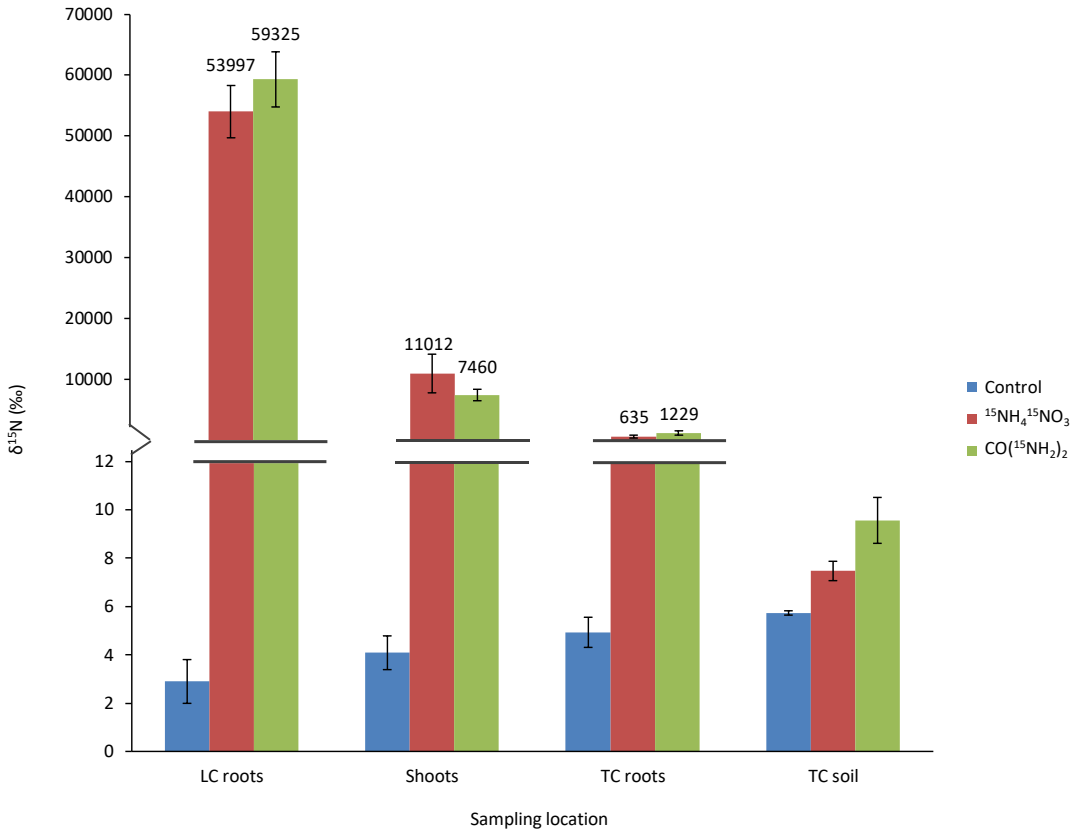

Figure A2.6. $\delta^{15}N$ values of different plant parts after application of the split-root labelling technique. White clover (*Trifolium repens*) plants were either labelled with DDW for the control, $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ (30 mM at 98 atom %) and sampled after 100h. LC- labelling compartment, and TC- transfer compartment. (mean ± standard error; n=5, without outliers removed as in Figure 3.16.)

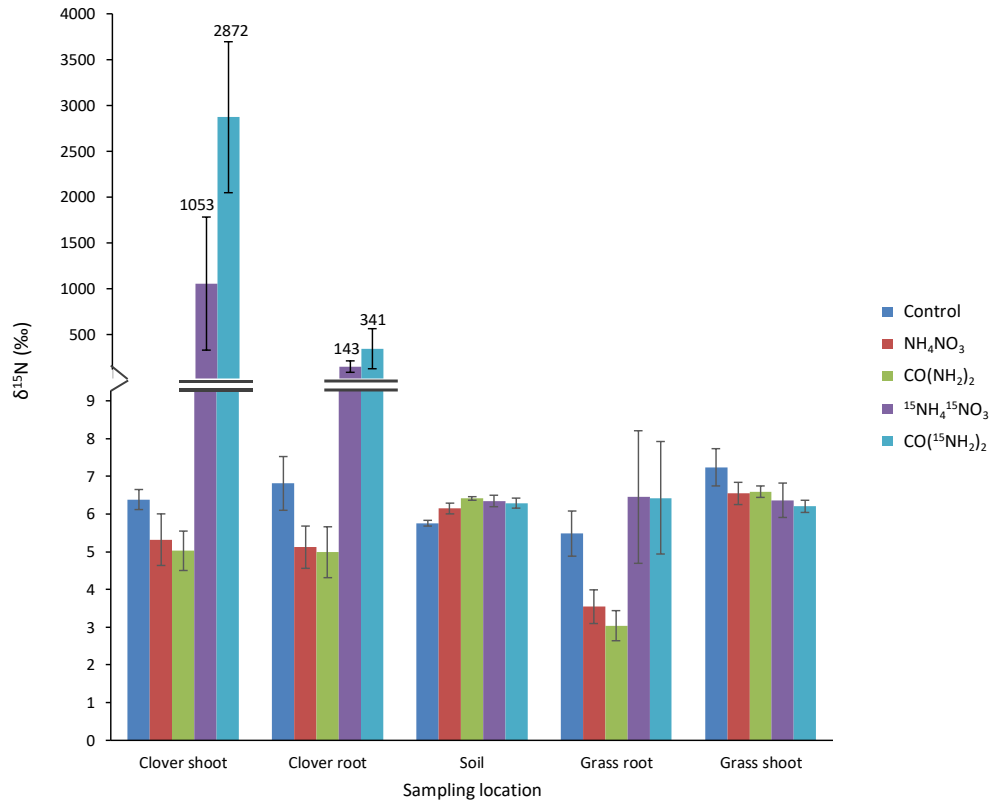

Figure A2.7. $\delta^{15}N$ values of different plant parts after application of the leaf-labelling technique to white clover (*Trifolium repens*) and determining uptake in ryegrass (*Lolium perenne*) with a 100 h labelling period (without outliers removed as in Figure 4.1). Leaves were either submerged in DDW for the control, natural abundance NH_4NO_3 or $CO(NH_2)_2$ or ^{15}N enriched NH_4NO_3 or $CO(NH_2)_2$. (mean ± standard error; n=4)

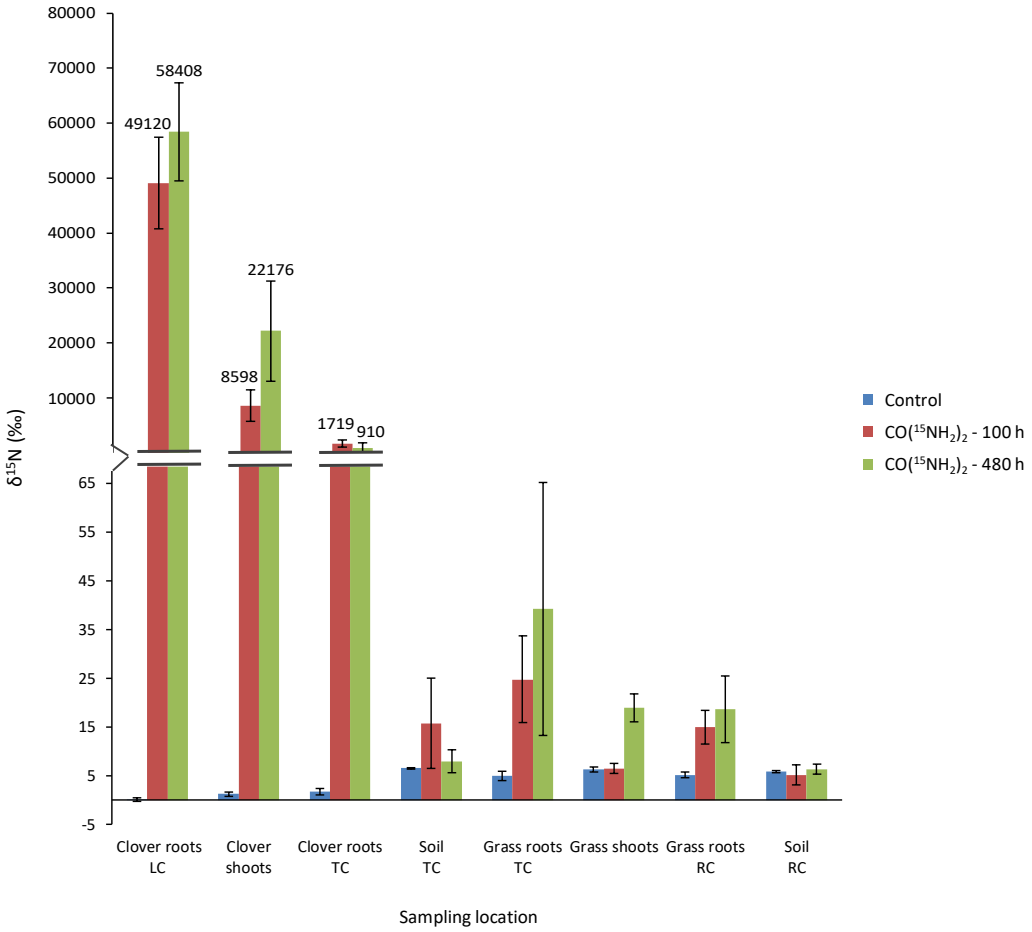

Figure A2.8. $\delta^{15}N$ values of different plant parts after application of the split-root labelling technique to white clover (*Trifolium repens*) and determining uptake in ryegrass (*Lolium perenne*) (without outliers removed as in Figure 4.2). Clover plants were either labelled with DDW for the control (sampled at 100 h), or ^{15}N enriched $CO(NH_2)_2$ and sampled after 100 h or 480 h. LC- labelling compartment, TC- transfer compartment and RC- receiving compartment. (mean ± standard error; n=4).

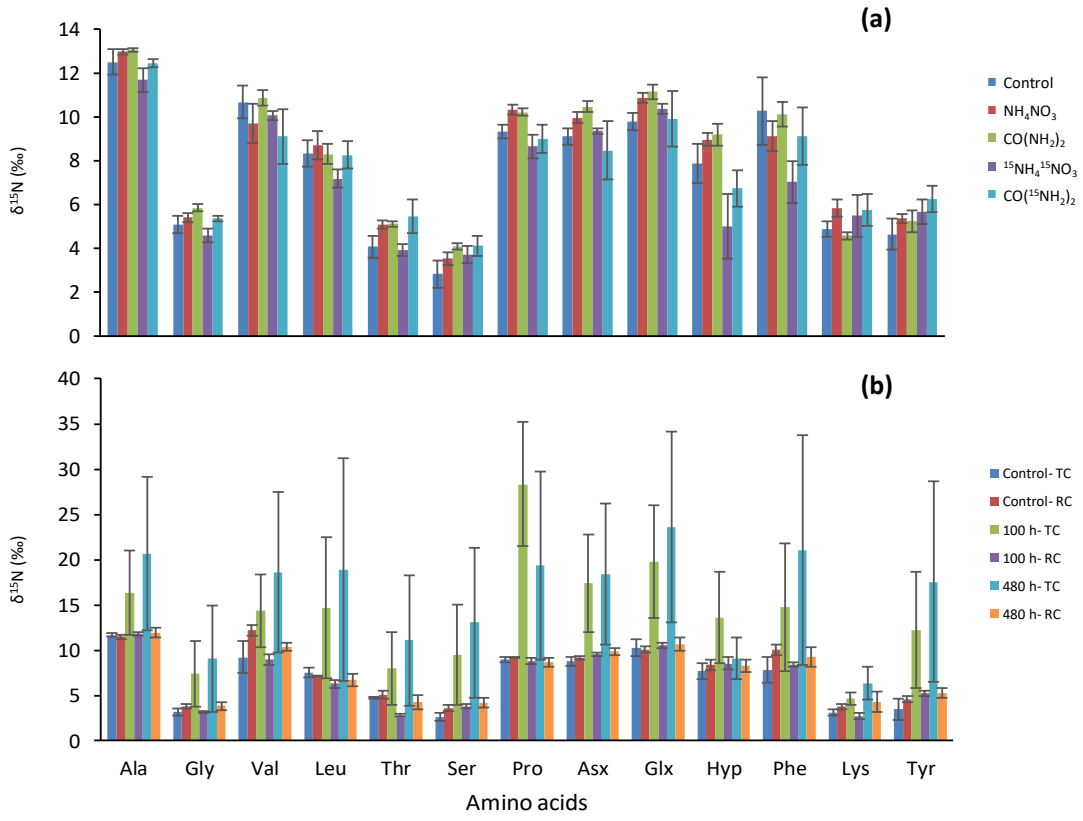

Figure A2.9. $\delta^{15}N$ values of individual hydrolysable soil AAs after application of ^{15}N-label to white clover (*Trifolium repens*) with associated ryegrass (*Lolium perenne*): (a) leaf-labelling technique. Leaves were either submerged in DDW for the control, natural abundance NH_4NO_3 or $CO(NH_2)_2$ or ^{15}N enriched $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ and harvested after 100 h. (b) Split-root labelling technique with DDW for the control (sampled at 100 h), or ^{15}N enriched $CO(NH_2)_2$ and sampled after 100 h or 480 h. TC- transfer compartment and RC- receiving compartment. (mean ± standard error; n= 4, without outliers removed as in Figure 4.4.)

Appendix A3

Tables comparing percentage incorporated and percentage retained of the applied ^{15}N-label into soil AAs

Table A3.1. Incorporation of the applied ^{15}N-label and retained ^{15}N in the bulk root incorporated into individual amino acids (%) in the roots of white clover (Trifolium repens) plants growing in rhizotrons after $^{15}NH_4{}^{15}NO_3$ application through different techniques. (Table 3.6 in thesis)

	% incorporated	% retained	% incorporated	% retained	% incorporated	% retained	% incorporated	% retained
	Spot		Multi		Spray		Leaf	
Alanine	27.0	59.6	37.3	90.3	11.5	48.8	3.6	35.3
Glycine	20.5	45.2	33.0	80.0	9.1	37.6	4.6	46.2
Valine	13.6	30.0	18.2	45.1	3.6	15.8	0.9	9.0
Leucine	24.6	54.1	25.5	62.3	6.9	29.4	3.4	37.2
Threonine	31.4	68.9	41.2	101.2	13.9	60.9	7.0	74.8
Serine	13.3	29.1	20.6	49.5	-	-	-	-
Proline	30.6	66.3	35.6	86.0	13.4	57.0	11.5	124.4
Aspartic acid	36.7	**80.1**	48.4	118.2	15.2	65.6	4.2	39.9
Glutamic acid	16.2	35.2	18.5	44.2	-	-	-	-
Hydroxyproline	**37.1**	79.9	42.9	104.0	18.6	79.2	**18.3**	**199.4**
Phenylalanine	16.4	36.0	12.6	31.0	4.8	20.8	4.6	46.7
Lysine	28.8	64.8	**49.7**	**121.5**	**22.9**	**96.7**	3.1	29.1
Tyrosine	13.9	30.3	10.1	20.1	2.7	11.6	1.9	18.8

TableA3.2. Incorporation of the applied ^{15}N-label and retained ^{15}N in the bulk transfer compartment soil incorporated into individual amino acids (%) following the application of $^{15}NH_4{}^{15}NO_3$ or $CO(^{15}NH_2)_2$ to white clover (Trifolium repens) in the labelling compartment. (Table 3.14 in thesis)

	% incorporated	% retained	% incorporated	% retained
	$^{15}NH_4{}^{15}NO_3$		$CO(^{15}NH_2)_2$	
Alanine	0.0025	76.63	0.0040	61.31
Glycine	0.0027	79.88	0.0037	57.83
Valine	0.0010	25.21	0.0014	17.94
Leucine	0.0014	42.81	0.0014	22.55
Threonine	0.0020	63.28	0.0022	34.42
Serine	0.0016	45.08	0.0022	34.31
Proline	0.0038	112.29	0.0121	187.87
Aspartic acid	0.0034	106.19	0.0036	56.65
Glutamic acid	0.0079	242.03	0.0107	158.92
Hydroxyproline	0.0003	6.30	0.0004	6.28
Phenylalanine	0.0005	12.99	0.0011	17.30
Lysine	0.0037	125.25	0.0018	26.25
Tyrosine	0.0006	19.01	0.0007	9.81

Table A3.3. Incorporation of the applied ^{15}N-label and retained ^{15}N in the bulk soil incorporated into individual AAs (%) for the split-root labelling technique. Maximum values are shown in bold. (Table 5.7 in thesis)

	No treatment		Clover incorporated		Clover exudates		Clover cut	
	% incorporated	% retained	% incorporated	% retained	% incorporated	% retained	% incorporated	% retained
Alanine	0.061	84.96	0.293	70.10	0.012	39.08	**0.022**	58.86
Glycine	0.056	76.72	0.334	78.92	0.010	16.74	0.020	53.10
Valine	0.030	42.04	0.129	29.20	0.005	8.76	0.008	19.24
Leucine	0.037	53.09	0.193	44.42	0.007	29.44	0.015	34.84
Threonine	0.054	62.27	0.189	44.14	0.010	25.00	0.011	28.09
Serine	0.038	51.54	0.165	37.70	0.007	18.40	0.008	20.92
Proline	0.039	54.67	0.218	53.35	0.007	19.77	0.020	51.18
Aspartic acid	0.059	79.79	0.236	53.48	0.011	25.02	0.011	28.18
Glutamic acid	**0.081**	**112.07**	**0.361**	**84.45**	**0.017**	**44.71**	0.020	**52.18**
Hydroxyproline	0.005	7.52	0.046	11.46	0.003	10.85	0.005	11.98
Phenylalanine	0.010	13.87	0.057	13.08	0.002	6.28	0.004	10.30
Lysine	0.029	34.31	0.142	33.22	0.006	47.61	0.006	16.79
Tyrosine	0.005	7.21	0.023	5.29	0.001	2.19	0.001	3.64
Total incorporation	0.494		2.384		0.098		0.151	

Table A3.4. Incorporation into individual AAs (%) of the applied ^{15}N label and retained ^{15}N in the bulk soil for the split-root labelling technique with ^{15}N enriched $CO(NH_2)_2$ only or ^{15}N enriched $CO(NH_2)_2$ with sterilised soil, weevil or fungi addition in the transfer compartment soil (TC). Maximum values are shown in bold (Table 6.8 in thesis)

	No treatment		Sterile		Fungi		Weevil	
	% incorporated	% retained	% incorporated	% retained	% incorporated	% retained	% incorporated	% retained
Alanine	0.0115	108.27	**0.0262**	**116.04**	0.0269	**139.63**	0.0295	100.42
Glycine	0.0112	107.66	0.0243	110.15	0.0257	132.66	0.0267	85.39
Valine	0.0055	52.42	0.0066	34.58	0.0058	26.72	0.0049	15.51
Leucine	0.0084	80.94	0.0121	56.45	0.0144	70.70	0.0133	50.09
Threonine	0.0080	78.12	0.0112	58.69	0.0104	55.66	0.0116	50.38
Serine	0.0069	68.51	0.0096	50.60	0.0103	60.50	0.0112	61.42
Proline	0.0144	133.37	0.0184	85.15	**0.0286**	139.33	**0.0341**	117.65
Aspartic acid	0.0118	114.77	0.0120	65.98	0.0108	52.44	0.0171	99.54
Glutamic acid	**0.0162**	**157.03**	0.0207	102.93	0.0233	117.51	0.0263	**157.75**
Hydroxyproline	0.0052	48.54	0.0048	25.20	0.0065	30.11	0.0071	29.77
Phenylalanine	0.0023	21.49	0.0031	14.67	0.0031	14.76	0.0023	9.26
Lysine	0.0035	30.98	0.0091	34.71	0.0065	35.72	0.0099	47.21
Tyrosine	0.0011	10.77	0.0020	10.32	0.0018	8.41	0.0019	6.41
Total incorporation	0.1059		0.1600		0.1739		0.1960	